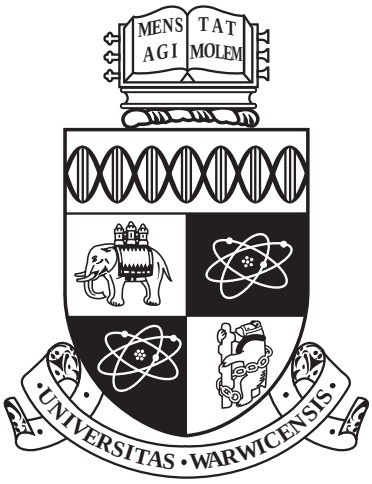

Control of Environmental Stress Responses by the Circadian Clock and Abscisic Acid

by

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Declarations

I declare that the work presented in this thesis was conducted by myself under the direct supervision of Doctor Isabelle Carré and Doctor Matthew Hannah, with the exception of those instances where the contribution of others has been specifically acknowledged. None of the work presented here has been previously submitted for any other degree.

Jack Cameron Grundy

Abstract

Plants are exposed to a variety of abiotic stresses, including salinity and drought. These environmental stresses cause major losses in crop yield. High salinity stress alone impairs crop production on at least 20% of irrigated land worldwide. Thus, the development of stress-tolerant crops is of major importance for food security. Many physiological responses to ensure acclimation to adverse environmental conditions require the synthesis and perception of the plant hormone abscisic acid (ABA). Recent studies have shown that the function of the circadian clock is altered under some abiotic stress conditions such as drought, and osmotic stress. The first part of this thesis investigates the role of the stress response hormone abscisic acid in changing the function of the clock under osmotic stress. It was found that multiple core clock genes are responsive to ABA application, with sharp transient induction of morning associated genes in particular. In comparison, osmotic stress caused a damping of the amplitude of gene expression. It was then shown that the disruptive effect of osmotic stress on circadian leaf movement rhythms required the biosynthesis of ABA. This is important as it demonstrates that ABA is a key factor in mediating osmotic stress responses to the clock. The second half of this thesis then focuses on how altered function of the clock might impact plant performance under drought or osmotic stress. It was found that the morning associated LATE ELONGATED HYPOCOTYL (LHY) transcription factor, which functions as a key component of the circadian clock, regulates many of the components of the ABA signalling pathway. Evidence was provided that, while overexpression of LHY results

in reduced ABA levels, ABA responsive gene expression is significantly increased upon ABA treatment. Finally, through phenotypic analysis it was determined that increased LHY expression leads to increased performance in drought and osmotic stress conditions. This is important as it suggests that manipulation of circadian clock function may be useful as a novel approach in the future engineering of stress tolerant crop lines.

Abbreviations

ABA Abscisic Acid

ABF ABA Responsive Element Binding Factor

ABI ABA Insensitive

ABRE Abscisic Acid Responsive Element

bZIP Basic Region/Leucine Zipper Motif

CAB Chlorophyll A/B Binding Protein

CBF C-Repeat/DRE-Binding Factor

CCA1 Circadian Clock Associated 1

cDNA complementary-DNA

ChIP Chromatin Immunoprecipitation

Col-0 Columbia ecotype of *Arabidopsis thaliana*

DRE Dehydration-Responsive-Element

DNA Deoxyribonucleic acid

EC Evening Complex

ECL Enhanced Chemiluminescence

EE Evening Element

ELF Early Flowering gene

GA Gibberellin (Gibberellic Acid)

Ler Landsberg ecotype of *Arabidopsis thaliana*

LHY Late Elongated Hypocotyl

LUX Lux Arrhythmo

ME Morning Element

mRNA Messenger RNA

MS0 Murashige and Skoog medium 0% Sucrose

PCR Polymerase Chain Reaction

PRR Pseudo-Response Regulator

qPCR Quantitative PCR

RGB Red Green Blue (additive colour model)

RNA Ribonucleic acid

RNAi RNA Interference

ROS Reactive Oxygen Species

SDS Sodium dodecyl sulphate

SWC Soil Water Content

TBST tris-buffered saline (TBS) and Polysorbate 20 (also known as Tween 20).

TOC1 Timing of CAB Expression 1

WS Wassilewskija ecotype of *Arabidopsis thaliana*

ZT Zeitgeber Time, time since dawn

Chapter 1

Introduction

1.1 The Circadian Clock

Circadian rhythms, from the Latin *circa* (about) and *dies* (day), are a subset of endogenous biological rhythms present in organisms across all kingdoms. To qualify as a circadian rhythm two requirements must be met. Firstly, the rhythm should generate a predictable output occurring at an interval of approximately 24 hours which persists under constant conditions, in a state known as 'free-running'. (McClung, 2008; Dunlap et al., 2004). Secondly, the rhythm must be susceptible to entrainment, the process by which external environmental signals act to synchronise the rhythms to the day-night cycle (Pittendrigh, 1979). This is an important feature as in order for the circadian clock to remain synchronised to the external environment across seasons, it is important that the rhythms are not hard-coded with fixed dawn and dusk times. Instead the system is plastic and responds to gradual, daily changes providing continuous re-entrainment, updating rhythms to match the current environment. The first experimental evidence of these rhythms came from Jean-Jacques de Mairan in 1792, who described the opening and closing of leaves in the plant *Mimosa pudica* and also showed that they persisted in constant darkness (De Marian, 1729). Persistence under constant conditions is a key differentiator

between circadian and diurnal regulation; the latter merely functioning as a reactionary response to external timing cues produced via the geological phenomenon of the Earth rotating about its axis.

Conceptually, circadian clocks can be described as a three-tier system (see Figure 1.1). In the first tier, environmental cues, often referred to as *zeitgebers* (German for time-givers) are perceived and then integrated into the central oscillator via input pathways. This ensures synchronicity with the external environment. The second tier contains the central oscillator, an autoregulatory timing circuit consisting of a network of interlocking negative transcription-translation feedback loops producing oscillating protein levels. Existence of multiple feedback loops has been hypothesised to increase the stability of the oscillator against parameter perturbations (Rand et al., 2004). The third tier contains the downstream signalling processes which are controlled by the central oscillator via output pathways. The characteristics of these outputs are discussed using a precise nomenclature (see Figure 1.1). Period refers to the time taken for one complete cycle. An output under circadian control will have a period of approximately 24 hours. Phase is used to describe the relationship between a point in a rhythm to a marker such as another rhythm. An example of this would be the peak expression of a gene in relation to dawn under day/night cycles. The amplitude of such a rhythmically expressed gene is described as half of the distance between the peak of expression and the trough.

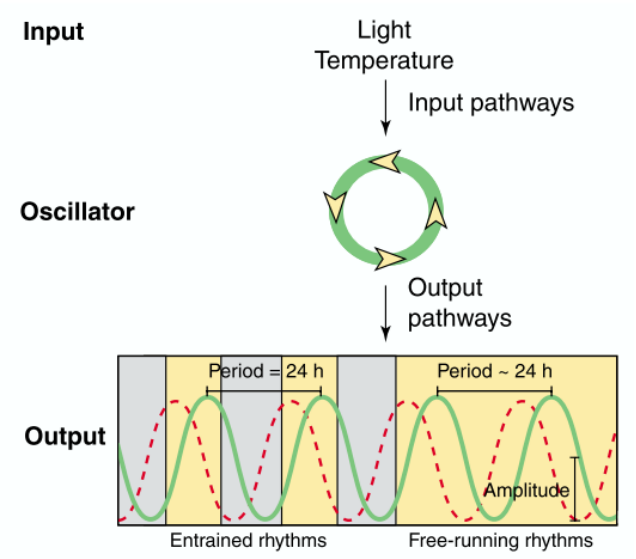

Figure 1.1 – Three-tier model of the *Arabidopsis* circadian clock. Environmental signals are perceived via input pathways and act to entrain the central oscillator. The central oscillator acts as a central control centre and drives rhythmicity of output pathways. Once entrained, oscillations persist in constant conditions a property known as 'free-running'. Yellow and grey boxes represent day and night respectively. Reproduced from (Barak et al., 2000).

Circadian rhythms have been found in both Prokaryotic and Eukaryotic organisms. Currently the only prokaryotic group known to exhibit true circadian rhythmicity is the cyanobacteria *Synechococcus* (Ishiura et al., 1998). However, rhythms have been observed in several single cellular eukaryotes such as the marine dinoflagellate *Lingulodinium polyedrum* also known as *Gonyaulax* (Roenneberg and Morse, 1993) and the fungi *Neurospora* (Gardner and Feldman, 1980; Bell-Pedersen, 2000) and *Aspergillus* (Greene et al., 2003). Many multicellular eukaryotes with circadian rhythmicity have been identified, including insects (Plautz et al., 1997), amphibians (Besharse and Iuvone, 1983), reptiles (Tosini and Menaker, n.d.), fish (Whitmore et al., 1998), birds (Zimmerman and Menaker, 1979) and mammals (Balsalobre et al., 1998; Welsh et al., 2004) including humans (Eckel-Mahan et al., 2012; O'Neill and Reddy, 2011; Roenneberg et al., 2007). Recent studies have also suggested circadian rhythmicity in archaea identifying rhythmic oscillations of peroxiredoxin oxidation

in *Halobacterium salinarum* and light dependent expression of cyanobacterial-like clock homologs (Edgar et al., 2012; Maniscalco et al., 2014). Currently, the only known organisms not to possess a circadian clock are those which inhabit environments lacking regular daily changes in light or temperature such as those at high latitudes or in deep caves (Lu et al., 2010; Moran et al., 2014).

The existence of multiple independent clock systems across kingdoms implies that the presence of such a mechanism is evolutionarily desirable and must increase fitness in those organisms that possess one. This is understandable as biological rhythms allow anticipation of changes in the external environment, something that is of particular importance to plants due to their sessile nature. For example it has been shown that *Arabidopsis* plants with arrhythmic clock mechanisms (due to overexpression of core clock gene *CCA1*) were not able to anticipate dawn under very short photoperiods (4L 20D). Whilst the wild type plants were able to survive (albeit with slowed growth due to very short-day conditions) the arrhythmic mutant began to die after 2 weeks with less than 5 % surviving for 4 weeks (Green et al., 2002). Plants produce more chlorophyll and fix more carbon when their endogenous period is matched to the environment and this effect is reflected in overall levels of biomass (Dodd et al., 2005). Studies have shown similar results in *Synechococcus* as strains with a functioning biological clock were found to outcompete those with defective clocks when grown in a rhythmic environment. This competitive advantage disappeared when grown in constant conditions (Woelfle et al., 2004).

Analysis of the key genes responsible for such rhythms in animals, fungi and plants has revealed very little conservation of core oscillator components suggesting that this time keeping mechanism has evolved independently multiple times (Eckardt, 2006; Dunlap et al., 2004). With the recent finding of rhythmic oxidation of peroxiredoxin (PRX) proteins in erythrocytes and conservation of PRX rhythms across

kingdoms (Humans, Mice, *Drosophila*, *Arabidopsis*, *Neurospora* and *Synechococcus*) it has been suggested that circadian rhythms may have originated as a selective advantage during the Great Oxidation Event. Rhythmic production of PRX may allow organisms to anticipate and mitigate the deleterious effects of high levels of reactive oxygen species (ROS) production driven by the solar cycle (O'Neill and Reddy, 2011; Edgar et al., 2012; Loudon, 2012).

Circadian rhythms are important in conferring fitness in humans too and are required in the co-ordination and homeostasis of the metabolism as well as the cognitive systems (Martinez-Lozano Sinues et al., 2014; Dibner et al., 2010; Muto et al., 2016; Ly et al., 2016). Disruptions to the circadian clock have been linked to the prevalence of breast, lung and colorectal cancer cases in shift workers (Blask et al., 2005; Reiter et al., 2006; Hansen, 2001; Papagiannakopoulos et al., 2016) as well as affective disorders such as depression (Benedetti et al., 2003; Salgado-Delgado et al., 2011). The circadian clock has also been linked to the ageing process in primates and humans; women with weaker circadian rhythms were found to have a higher mortality risk (Tranah et al., 2011; Zhdanova et al., 2011).

Not only does circadian disruption lead to medical issues but it can also affect the efficacy of drugs given in response as treatment (Levi, 2009; Dallmann et al., 2016; Ahrén et al., 2013; Kim et al., 2013; Innominato et al., 2011; Guo et al., 2015). Albumin and other plasma binding proteins are subject to circadian regulation meaning that free-drug levels can be affected depending on the time of administration. Many cytochrome P450 enzymes, which are important in the first phase of drug metabolism, have circadian controlled expression patterns (Košir et al., 2013; Tomalik-Scharte et al., 2014). With such temporal regulation of enzyme levels the administration of drugs has to be co-ordinated in order to maximise effectiveness or so as to prevent increased toxicity.

1.1.1 Plant circadian clock

Whilst circadian clock genes have been identified in many plants the higher plant *Arabidopsis thaliana* is by far the most widely studied. Although some details remain to be confirmed, the understanding of the core circadian architecture is well established (see Figure 1.2) (Matsushika et al., 2007; Gendron et al., 2012; Carré and Veflingstad, 2013; Adams et al., 2015). A large proportion of the *Arabidopsis* genome is under circadian control. Early analyses revealed circadian control of 10-15 % of transcripts (Harmer et al., 2000; Schaffer et al., 2001) whilst a recent meta analysis of multiple microarrays has shown that as much as 30-40 % of expressed genes are regulated by the circadian clock, and up to 80 % of the genome displays rhythmicity under some type of diurnal environmental cycle (Covington et al., 2008). This allows downstream responses in the plant such as DNA repair, metabolite biosynthesis and photosynthesis to be regulated via the clock (Nagano et al., 2012).

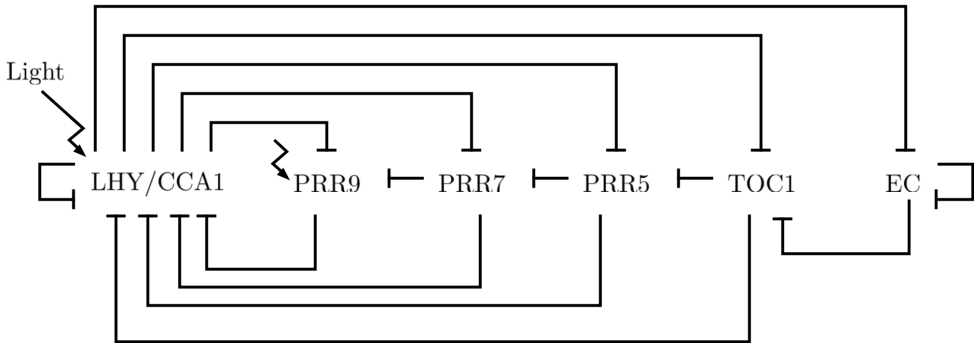

Figure 1.2 – Schematic depicting the central clock model in *Arabidopsis*. *LHY* and *CCA1* are expressed in the morning and act to repress the *PRR* genes as well as the Evening Complex (EC). LHY and CCA1 also target their own promoters and repress their own transcription which in turn relieves the repression on the *PRR* genes and EC. The PRR genes maintain repression on *LHY* and *CCA1* during the afternoon and evening. The EC can then represses the expression of the *PRRs* during the night which allows *LHY* and *CCA1* to be transcribed the following dawn and the cycle restarts. Acute transcriptional responses to light are indicated by lightning symbols.

Circadian clocks among organisms share similar mechanisms of interlocking positive

and negative transcription and translation feedback loops (TTFL) between their core circadian clock genes, providing rhythmicity to the rest of the genome. In the *Arabidopsis* oscillator, *LATE ELONGATED HYPOCOTYL (LHY)* and *CIRCADIAN CLOCK ASSOCIATED 1 (*CCA1), two closely related MYB-domain transcription factors, are expressed simultaneously at dawn with acute transcriptional induction in response to light (Kim et al., 2003) (see Figure 1.2). Transcription levels of *LHY* and *CCA1* peak shortly after dawn, with peak protein levels occurring approximately 2 hours after (Wang and Tobin, 1998; Schaffer et al., 1998). Double loss of function mutants *lhy/cca1* confirmed a central role in the clock for both of these genes (Alabadí et al., 2002; Mizoguchi et al., 2002). Overexpression of *CCA1* resulted in reduced and arrhythmic expression of both itself and *LHY* (Wang and Tobin, 1998) whilst overexpression of *LHY* was shown to abolish rhythmic expression of other clock genes as well as its own endogenous expression pattern (Schaffer et al., 1998). *LHY* and *CCA1* have partially redundant function as neither were able to entirely compensate for the loss of the other, producing short-period rhythms (Mizoguchi et al., 2002; Green and Tobin, 1999).

LHY/CCA1 were thought to activate *PSEUDO RESPONSE REGULATOR 9 (PRR9)* and *PSEUDO RESPONSE REGULATOR 7 (PRR7)* (Farré et al., 2005). However it has now been shown that *LHY* binds to and represses expression of PRR9, 7, 5 and *TIMING OF CAB EXPRESSION 1 (PRR1/TOC1)* (Adams et al., 2015), whilst *CCA1* has been shown to repress *PRR5* (Kamioka et al., 2016). At the same time *LHY/CCA1* represses the Evening Complex (EC), a protein complex formed by *EARLY FLOWERING 3 (ELF3)*, *EARLY FLOWERING 4 (ELF4)* and *LUX ARHYTHMO (LUX)* (Alabadi, 2001; Nusinow et al., 2011; Lu et al., 2012; Adams et al., 2015).

As LHY/CCA1 also bind their own promoters and repress their own expression,

this allows for the PRR genes to be expressed during the afternoon resulting in sequentially increased levels of *PRR9, 7, 5* and finally *TOC1* transcription which further repress *LHY/CCA1* transcription through the evening and night (Adams et al., 2015; Nakamichi et al., 2005, 2010). With reduced *LHY/CCA1* expression, the repression of the Evening Complex is also removed. The EC can then represses the expression of the *PRRs* causing the repression of *LHY/CCA1* to taper off during the night period (Strayer et al., 2000; Matsushika et al., 2000). With reduced PRR levels, the morning associated genes can again be transcribed the following dawn and the cycle restarts.

As stated previously, it is vital that the endogenous timing mechanism is synchronous with the external environment and this requires continual re-entrainment of the rhythm. For *Arabidopsis* the most important environmental cue for entrainment is light. Light sensing is not merely a binary presence or absence detection, light quality affects entrainment and under low intensity the circadian period is lengthened (Somers, Webb, Pearson and Kay, 1998). Plants detect light through three distinct families of photoreceptors; phototropins, cryptochromes and phytochromes. The two most important of these, in terms of circadian entrainment, are the cryptochromes (detecting blue light) and phytochromes (detecting red and far red light), which have both been shown to act as clock modulating sensory input components (Somers, Devlin and Kay, 1998). The cryptochrome family consists of two members, (CRY1 and CRY2), which share sequence identity to type II DNA photolyases from which they are thought to have evolved and act redundantly in blue light input to the clock (Ahmad and Cashmore, 1993; Guo et al., 1999; Devlin and Kay, 1999). They contain two chromophores, a flavin adenine dinucleotide (FAD) and a pterin methyltetrahydrofolate (MTHF) and are constitutively located in the nucleus (van der Schalie and Green, 2005). Double mutants, *cry1cry2* , have been shown to maintain robust rhythmic *CAB2*::*luc* bioluminescence under free running conditions

(Devlin and Kay, 2000).

The Phytochrome family consists of five members (PHYA - E) which encode homodimers consisting of two polypeptide chain apoproteins that are covalently bound to a tetrapyrrole chromophore (Clack et al., 1994; Murgida et al., 2007). Phytochromes exist in two photoconvertible forms, an inactive form which can absorb red light (Pr), and an active form which can absorb far red (Pfr). Absorption of red light produces the active form Pfr which can enter the nucleus, where it acts as a transcriptional regulator by binding to phytochrome-interacting factor 3 (PIF3), a basic-helix-loop-helix (bHLH) transcription factor that targets the G-box motif (Martnez-Garca et al., 2000; Quail, 2010; Soy et al., 2012). *CCA1* was shown to be induced in response to red light via phytochrome signalling and *luciferase* assays revealed that phyB played an important role in regulating light input into the circadian clock as *phyB* mutants lengthened period and phyB overexpression shortened period relative to the wild type (Wang and Tobin, 1998; Somers, Devlin and Kay, 1998). It was also shown that phyA (which detects low fluence red and blue light) and phyB (which detects high fluence red light) act additively to regulate light input to the clock. However, phytochromes exhibit some degree of redundancy as *phyA,B, cry1,2* quadruple mutants still maintain robust circadian leaf movement rhythms under white light (Yanovsky et al., 2000). This implies that phyC, D and E are able to mediate light signals to the clock sufficiently for entrainment. Quintuple phytochrome mutants *phyABCDE* have arrhythmic circadian leaf movement under red light (Strasser et al., 2010).

One may assume that if organisms are able to sense the environment around them, they are merely responding in a reactionary manner to stimuli. However, entrainment to the environment allows anticipation of recurrent events, such as dawn and dusk, allowing the scheduling of physiological and metabolic processes over the

course of 24 hours. For example, the genes encoding the chlorophyll-a/b binding proteins (cab) of the light harvesting complexes are shown to have rhythmic expression with peak levels occurring at ZT3-8 and the minimum levels at ZT10-16 (ZT, zeitgeber time, time from subjective dawn, dawn = ZT0). Transcript levels of *CAB* begin to rise approximately 3 hours before the onset of dawn allowing sufficient time for the transcription, translation, localisation and assembly into operational photosynthetic machinery to capture the first light at dawn and maximise the amount of light energy harvested across the day (Millar and Kay, 1991; Kay, 1993).

1.2 Plant Responses to Abiotic Stress

Abiotic stress is defined as the negative impact of non-living factors on a living organism in a specific environment. For example, water deficit (osmotic or drought stress) or extremes of temperature. Abiotic stress can affect cellular homeostasis via a variety of mechanisms. Freezing temperatures promote intra- and extra-cellular ice crystal formation, leading to cellular damage, dehydration and disruption of the plasma membrane (Steponkus and Webb, 1992; Pearce, 2001; Yamazaki et al., 2008, 2009). High temperatures damage the plasma membrane by increasing its fluidity, leading to ion leakage. Heat stress can also result in inhibition of enzymatic activity and photosynthesis (Salvucci and Crafts-Brandner, 2004; Sharkey, 2005; Wahid et al., 2007; Allakhverdiev et al., 2008). High temperatures, salinity and drought stress are often accompanied by production of reactive oxygen species (ROS) which can lead to denaturation of functional and structural proteins (Gill and Tuteja, 2010; Suzuki et al., 2012).

Abiotic stress and the consequent effects on crop yield are a major problem within

the agricultural industry. As recently as 2012, the USA suffered its most severe drought since 1956 with 80 % of crop growing territory being subjected to some level of drought (Eischeid et al., 2014; Joo et al., 2016). Europe is also affected by water deficit with estimates of €100 billion in economic damage caused by drought over the last 30 years (as of 2007). This is a figure which is set to rise, with the number of areas affected by drought having already risen by 20 % between 1976 and 2006 (European Union, 2007). In addition to the direct loss for the companies and farmers involved there is also a knock-on effect to the general public with increased market prices and increased taxation in order to subsidise the companies' losses and ensure continued food production the following season.

In times of environmental stress, such as drought, stomata close (Leung and Giraudat, 1998) reducing transpiration and the loss of moisture to the air (Koornneef et al., 1998). Plant growth is arrested and lateral root formation is also inhibited but root hydrotropism is promoted in an attempt to penetrate the soil deeper in search of moisture (Antoni et al., 2013; Skirycz and Inze, 2010). Osmolytes such as proline are produced to create a competitive osmotic gradient with the surrounding soil, whilst LEA proteins are produced as osmoprotectants (Liang et al., 2013; Wang et al., 2014; Hand et al., 2011). Leaves may curl to produce small micro-climates immediately surrounding the leaf cuticle with the aim to contain some moisture and reduce transpiration further (Kadioglu et al., 2012). If water-stress persists long term leaf surface area will decrease to reduce transpirational loss and eventually leaves will be completely abscised. This is a last resort which not only results in the inability to photosynthesise but will also result in plant death should drought conditions persist.

1.2.1 Abscisic Acid (ABA) as a key hormone

Abscisic acid (initially named abscisin-II) was first isolated from *Gossypium hirsutum* (cotton) in 1965, when compounds responsible for leaf abscission were being isolated (Ohkuma et al., 1965; Cornforth et al., 1965*b*). Subsequently, ABA was also shown to play a role controlling seed germination. ABA acts antagonistically to gibberellic acid and promotes dormancy in seed. For this reason it was also named dormin. After dormin and abscisin II were found to be the same they were renamed abscisic acid (ABA) (Cornforth et al., 1965*a*, 1966). ABA can exist in four stereoisomers, although the active isomer found naturally in plants is (+)-2-*cis*-4-*trans*-ABA with a very small ratio of inactive (+)-2-*trans*-4-*trans*-ABA which is formed in a reversible isomerisation upon exposure to UV light.

ABA is constitutively present at low levels and is important for normal development in plants but is highly upregulated in response to drought and osmotic stress (Creelman and Zeevaart, 1985). This response is crucial to plant survival in such conditions. This was demonstrated in mutant lines defective in either ABA biosynthesis or ABA perception (Koornneef et al., 1998). For example mutation of ABA biosynthesis genes such as *aba1*, *aba2* and *aba3* increase plant sensitivity to drought, as the stress response cannot be initiated (Koornneef et al., 1998).

Biosynthesis of ABA occurs in plastids and requires synthesis of C_{40} carotenoid products (β-carotene, phytoene, ζ-carotene and lycopene) as precursors which can then feed into the ABA dedicated pathway (see Figure 1.3). The first step of the dedicated ABA synthesis pathway is a two-step epoxidation of zeaxathin to all-trans-violaxathin that is catalysed by zeaxanthin epoxidase (ZEP). 9'-cis-epoxycarotenoid dioxygenase (NCED) then catalyses the oxidative cleavage to form xanthoxin. Three possible pathways can then lead to the production of ABA although the most common is conversion of xanthoxin to abscisic aldehyde (ABAId) via short chain dehy-

drogenase/reducatse (SDR) and then oxidation of ABAld to form ABA via aldehyde oxidase (AO) (Seo, 2002; Finkelstein, 2013). ABA can be catabolised via 8'-hydroxylation by the cytochrome P450 member CYP707A to yield phaseic acid (Saito et al., 2004).

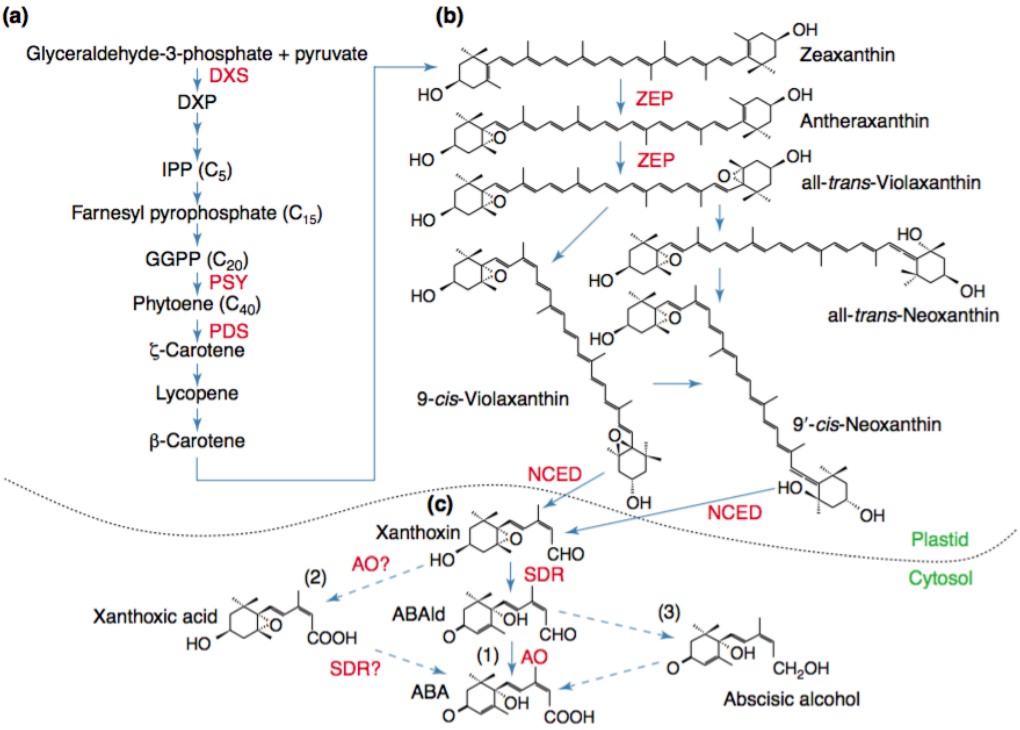

Figure 1.3 – Schematic depicting the biosynthesis pathway for abscisic acid in *Arabidopsis* a) C_{40} carotenoid products (β-carotene, phytoene, ζ-carotene and lycopene) feed into the dedicated ABA biosynthesis pathway. **b)** Zeaxanthin epoxidase (ZEP) catalyses zeaxanthin to all-trans-violaxanthin via a two-step epoxidation. **c)** It then undergoes oxidative cleavage to form xanthoxin, catalysed by 9'cis-epoxycarotenoid dioxygenase (NCED). Xanthoxin can then be converted to ABA by three distinct routes forming either xanthoxic acid or abscisic alcohol as intermediates. The most common route is into abscisic aldehyde (ABAId) via short chain dehydrogenase reducatse (SDR) and finally to abscisic acid via oxidation of ABAId by aldehyde oxidase (AO). Reproduced from (Seo, 2002).

Due to tissue specific differences in synthesis and catabolism rates many papers merely state that the basal level of ABA is low or in the nano-molar range (Umezawa et al., 2006). It is agreed, however, that ABA levels increase about 10-fold within 3 - 4 hours in response to drought (Zeevaart, 1980; Creelman and Zeevaart, 1985; For-

cat et al., 2008). ABA is predominantly synthesised within the vascular tissues and exported to the extracellular apoplastic space via an ATP-binding cassette (ABC) transporter (Kuromori et al., 2010). ABA can then be actively imported into cells at the sites of ABA action (such as guard cells, roots and leaves) via another ABC transporter (Kang et al., 2010). However a second class of ABA importer exists and unexpectedly, is primarily located in the vascular tissue (Kanno et al., 2012). This suggests that ABA might be imported back to the site of biosynthesis to regulate production.

A number of putative ABA binding proteins have been reported but their roles as receptors in ABA signalling have been disputed. ABA-binding protein 1 (ABAP1), discovered in barley aleurone via an antibody screen (Razem et al., 2004) was revealed via sequence analysis to be related to *Arabidopsis* Flowering Time Control Protein A (FCA), an RNA binding protein. An initial report demonstrated FCA binding with ABA, however this was later retracted after attempts to reproduce the data failed and a calculation error was discovered in the original analysis (Risk et al., 2008; Razem et al., 2008). An ABA binding protein was then discovered in *Vicia faba* (broad bean) (Zhang et al., 2002). The *Arabidopsis* homologue was named ABAR for (ABA receptor) and is also referred to as H SUBUNIT OF THE MAGNESIUM-PROTOPORPHYRIN IX CHELATASE/GENOMES UNCOUPLED 5 (ABAR/CHLH/GUN5) (Shen et al., 2006). The only known binding proteins shown to function in ABA signalling are those of the Pyrabactin Resistance (PYR) / Pyrabactin Resistance-Like (PYL) / Regulatory Component of ABA of ABA Receptor (RCAR) family (Ma et al., 2009; Nishimura et al., 2010; Park et al., 2009; Santiago, Rodrigues, Saez, Rubio, Antoni, Dupeux, Park, Márquez, Cutler and Rodriguez, 2009). The PYR/PYL family belong to a broader class of soluble, ligand binding proteins known as the steroidogenic acute regulatory protein, (StAR)-related lipid-transfer (START) domain superfamily. START-domain mem-

bers contain a conserved helix-grip domain that forms a hydrophobic biding pocket. ABA is known to bind to this domain in the PYR/PYL proteins (Cutler et al., 2010; Santiago, Dupeux, Round, Antoni, Park, Jamin, Cutler, Rodriguez and Márquez, 2009).

The PYR/PYL family contains 14 members and all but PYL13 are able to activate ABA responsive gene expression. The family is further divided based on oligomeric state, PYR1 PYL1 and PYL2 are dimeric whilst PYL5, 6 and 8 are monomeric. This results in a difference in binding affinities, with the monomeric receptors binding more strongly (Antoni et al., 2013; Gonzalez-Guzman et al., 2012; Zhang et al., 2013). Despite these differences there is a large degree of redundancy between the receptors. A quadruple mutant *pyr1pyl1pyl2pyl4* was required to produce a mild ABA insensitive phenotype whilst sextuple mutant *pyr1pyl1pyl2pyl4pyl5pyl8* had an ABA insensitivity at least 1 order of magnitude greater than the quadruple mutant (Park et al., 2009). The spatial expression of these receptors is tissue specific, PYL3, 10 -13 were only detected at very low levels in whole genome microarrays. PYR1 and PYL1-9 were much more highly expressed in roots, young and old leaves and stem tissue although levels fluctuated between different tissues (Gonzalez-Guzman et al., 2012).

The signalling pathway downstream of PYR/PYL receptors is shown in Figure 1.4. ABA responsive transcriptional regulators such as ABFs, ABI3 and ABI5 require phosphorylation from Sucrose non-fermenting-1 (SNF1)-related protein kinases (SnRKs) in order to bind to ABA responsive Elements (ABRE: ACGTGG/TC) and induce ABA responsive gene transcription. SnRKS are held inactive by the phosphatase action of group A protein phosphatases (PP2Cs). Binding of ABA causes a conformational change in two loops surrounding the binding pocket of the PYR/PYL named the 'gate' and 'latch' loops. This creates a binding surface which

can then interact with PP2Cs including: Hypersensitive to ABA 1 (HAB1), ABA-Insensitive 1 (ABI1), ABA-Insensitive 2 (ABI2) and PP2CA. These act as negative regulators of the pathway, de-phosphorylating SnRK2.2, 2.3 and 2.6/OST1 thus reducing their catalytic activity. Once the PYL-ABA complex is bound to the PP2C the phosphatase action is inhibited, removing the negative regulation which otherwise keeps the SnRK2.2 kinases in an inactive state. With a phosphorylated serine residue (Ser^{175}), SnRK kinases are now able to phosphorylate target ABFs, ABI3 and ABI5 and in turn activate ABA-dependent stress responses (Xiong and Zhu, 2003; Cutler et al., 2010; Huang et al., 2007; Raghavendra et al., 2010; Miyakawa et al., 2013; Weiner et al., 2010)

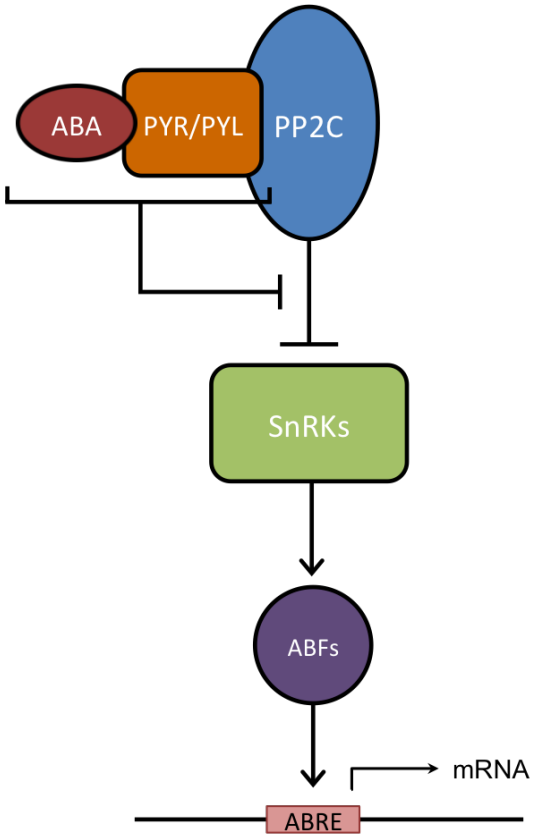

Figure 1.4 – Schematic depicting the ABA signalling pathway in *Arabidopsis*. Without the presence of ABA, the group A protein phosphatases (PP2Cs) dephosphorylate Sucrose non-fermenting-1 (SNF1)-related protein kinases (SnRKs), thus preventing activation of ABRE-binding factors (ABFs). When ABA is present it binds to a member of the Pyrabactin Resistance / Pyrabactin Resistance-Like / Regulatory Component of ABA of ABA Receptor (RCAR) family. This complex can then bind to a PP2C, inhibiting its phosphatase action. This inhibition allows SnRKs to become phosphorylated and to activate ABFs. ABFs can bind to ABRE target motifs and drive ABA responsive gene expression.

1.2.2 ABA-independent stress responses

Plant responses to abiotic stress are brought about via a combination of downstream signalling from both ABA-independent and ABA-dependent pathways (see Figure 1.5). Evidence for ABA-independent stress responses originated from the observation that ABA-inducible genes were induced by both cold and drought treatments in mutants unable to biosynthesise ABA. These genes included *RESPONSIVE TO DESICATION 29A* (*RD29A*) (Yamaguchi-Shinozaki and Shinozaki,

1994; Nakashima et al., 2006; Horvath et al., 1993), *LOW TEMPERATURE INDUCED 40* (*LTI40*) (Nordin et al., 1991) and *COLD REGULATED 6.6 and 4.7 (COR6.6 and 47)* (Iwasaki et al., 1997). Analysis of the promoter regions of these genes revealed a conserved promoter motif (A/GCCGA), named the dehydration-responsive-element / C-repeat (DRE/CRT) (Yamaguchi-Shinozaki and Shinozaki, 1994). This motif has since been reported in both drought and cold responsive genes. Two transcription factor groups, DREB1/CBF and DREB2, members of the larger AP2/ERF family, were found to interact with this motif in *Arabidopsis* (Liu et al., 1998). These transcription factors are highly conserved and homologs have been identified in a wide range of plant species such as *Hordeum vulgare* (barley) (Xue and Loveridge, 2004), *Oryza sativa* (rice) (Dubouzet et al., 2003) and *Zea mays* (maize) (Qin, 2004; Qin et al., 2007). This would suggest that ABA-independent stress responses are important in conferring plant fitness. Experiments over expressing DREB1 and DREB2 homologs have demonstrated this with increased performance in drought, osmotic stress and cold conditions in *Arabidopsis* (Shinozaki and Yamaguchi-Shinozaki, 2000). Despite increased stress tolerance over expression produced severe growth retardation. This was mitigated when over expression was driven under the *RD29A* promoter (Kasuga et al., 1999). Similarly over expression of DREB/CBF homologs in crop species such as *Triticum aestivum* (wheat) (Pellegrineschi et al., 2011), *Oryza sativa* (Ito et al., 2006; Datta et al., 2012), and *Glycine max* (soybean) (Polizel et al., 2011; de Paiva Rolla et al., 2013) were shown to enhance drought tolerance. Over expression of DREB2 has shown minimal improvement in freezing tolerance but greater improvement in plant performance in response to dehydration (Sakuma et al., 2006). These results have led to the understanding that whilst drought stress responses are co-regulated with cold responses via DREB1/CBF, heat stress is likely to be preferentially mediated via DREB2.

Another transcription factor family, NAC (containing the NAC domain: NAM (no apical meristem), ATAF1/2 and CUC2 (cup-shaped cotyledon)), has been shown to have important functions in regulating ABA-independent stress responses. They bind to a NAC-recognition sequence (NACR) with a core motif sequence (CACG), activating downstream drought inducible *EARLY RESPONSE TO DEHYDRATION (ERD1)* (Tran et al., 2004). Overexpression of these transcription factors has also led to increased performance under drought conditions in *Oryza sativa* (rice) (Hu et al., 2006).

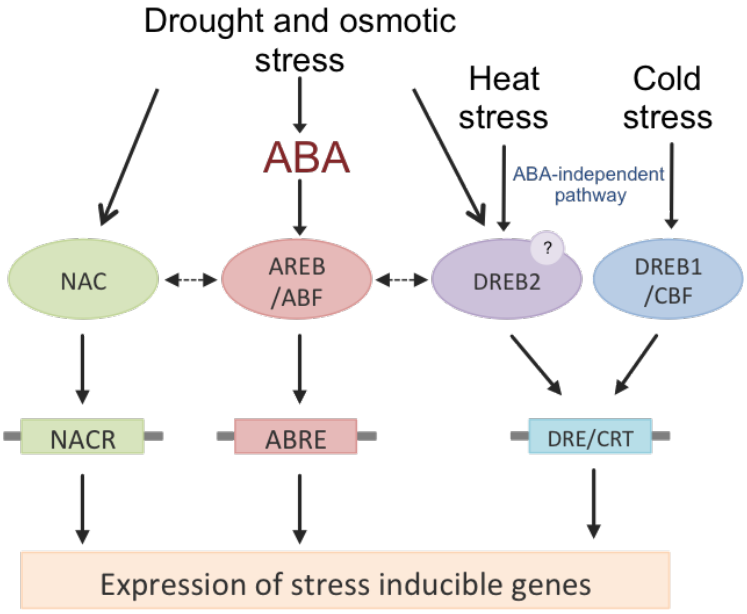

Figure 1.5 – Abiotic stress responsive transcription pathways. Schematic depicting the relationship between ABA-dependent and ABA-independent signalling pathways and how they correlate with different abiotic stress stimuli. Dotted lines indicate cross talk. DREB2 contains a small question mark to indicate that it is not yet confirmed to only regulate heat stress responses. Adapted from (Nakashima et al., 2014).

Recent studies have begun to show that many stress responsive genes are circadian regulated (see Figure 1.6) and may act to alter the function of the circadian clock. Stress related motifs have been found in the soybean and barley clock gene homologs (Bieniawska et al., 2008; Habte et al., 2014; Campoli et al., 2012). This may allow reprogramming of the circadian clock in response to abiotic stress stim-

uli. For example high salinity in soil increased the circadian period in winter wheat (Erdei et al., 1998). In barley roots, osmotic stress increased the expression levels of clock genes in comparison to control conditions. Expression of evening-expressed clock genes was increased and also phase shifted, advancing their peaks of expression (Habte et al., 2014). Similarly, drought stress in soy bean reduced the expression of evening-expressed circadian genes (*TOC1*, *LUX*, *ELF4* and *PRR*s) which disrupted the circadian system (Marcolino-Gomes et al., 2014).

Although the circadian clock is temperature compensated, detection of large temperature changes and temperature shock do affect the function of the circadian clock in many organisms. Cold stress dampens the amplitude of *Arabidopsis* circadian clock oscillations (Bieniawska et al., 2008) although this seems to be dependent on the severity of the stress. Short term (4-20 hours) cold stress was shown to induce expression of most core clock genes whilst severe cold stress (exposures longer than 24 hours) resulted in low amplitude oscillations. For example, *TOC1* had low amplitude of expression although expression levels remained high, whereas the peak expression levels of *LHY*, whilst still robust, were 4-8 fold lower (Bieniawska et al., 2008). On the other hand, *LUX* expression maintained a high amplitude of expression and has since been shown to be directly regulated via the ABA-independent abiotic stress response pathway with CBF1 binding to a CRT/DRE motif within the *LUX* promoter sequence to increase expression levels (Chow et al., 2014).

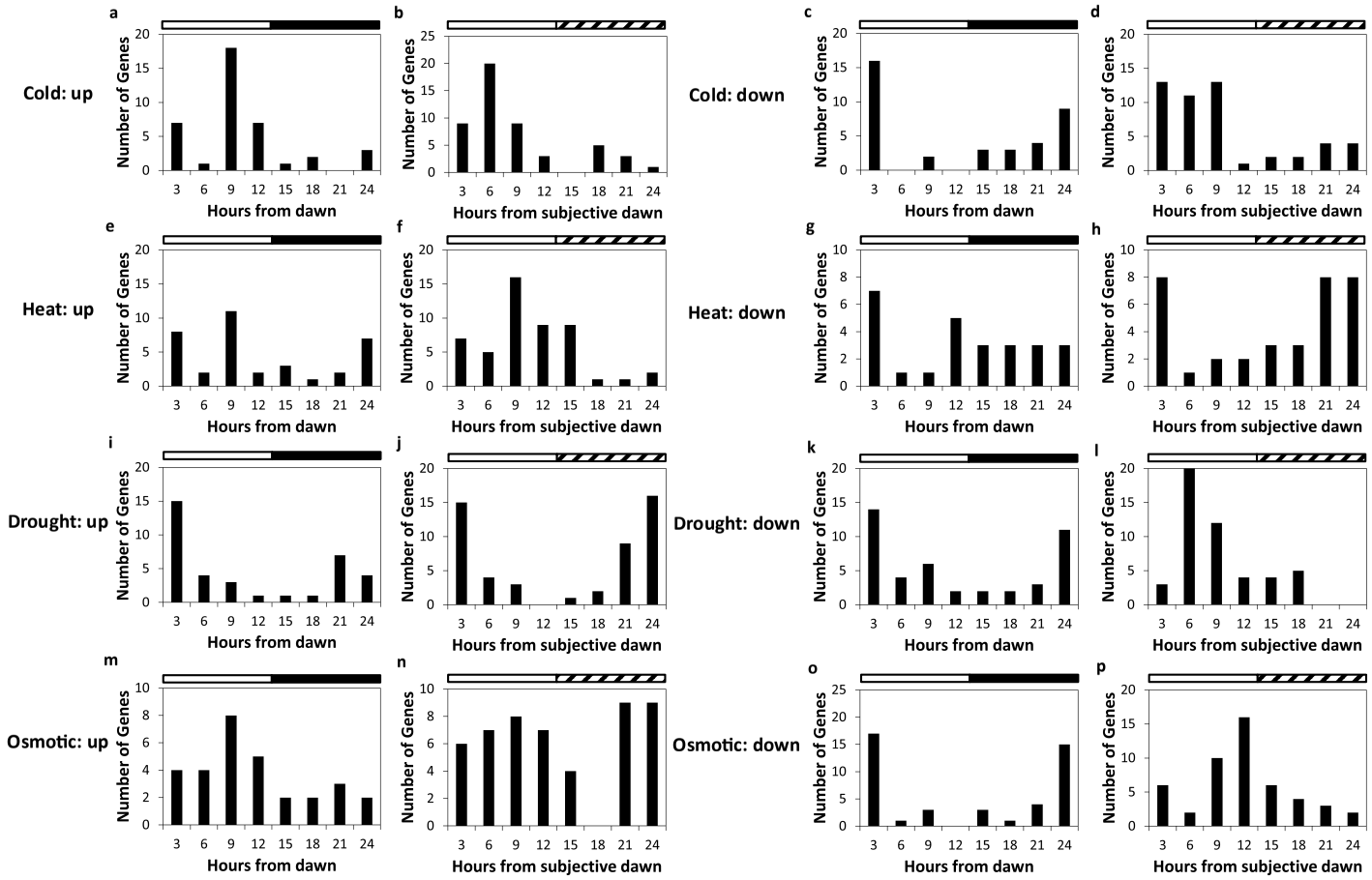

Figure 1.6 – Circadian regulation of cold, heat, drought and osmoticum-responsive genes in *Arabidopsis*. Histograms show the timing of peak mRNA levels in 12L12D or following transfer to constant light, for the 50 most significantly stress-induced (up) or repressed (down) genes that also exhibit circadian regulation. **a-d)** Cold, **e-h)** heat, **i-l)** drought and **m-p)** osmotic stress. Lists of stress-responsive genes were based on the following datasets: 24 h at 0C (Lee et al., 2005), 3 h of at 38C (Barah et al., 2013), water limitation for 4 days (Wilkins et al., 2010), and growth on 300 mM mannitol (Kilian et al., 2007). Phase data for Arabidopsis genes were obtained from experiments LDHH-Stitt and LL12 in the Diurnal database (Blasing et al., 2005; Mockler et al., 2007; Covington et al., 2008). Solid white and black bars indicate intervals of light and darkness, respectively, whereas hatched bars indicate subjective nights. Reproduced from (Grundy et al., 2015).

1.3 Circadian Regulation of ABA Responses

Diurnal variations in ABA production have been reported in numerous species. *Arbutus unedo* (Strawberry-tree) has its highest concentrations peak during early morning and early afternoon (Burschka et al., 1983). Phytochrome mutants have shown that light stimulates the degradation of ABA in *Nicotiana plumbaginifolia* and that the initial morning peak is only short lived (Kraepiel et al., 1994). In *Nicotiana tabacum* ABA levels peak at the beginning of the dark phase, although a transient induction of ABA was seen at dawn along with an "after midday" peak (Nováková et al., 2005). In addition to this, studies are revealing circadian control of ABA biosynthesis. For instance, NCED is diurnally regulated with transcripts peaking at the end of the day (Thompson, Jackson, Parker, Morpeth, Burbidge and Taylor, 2000). More than 40 % of ABA induced genes (492/1194) are circadian regulated, which is a significant overrepresentation relative to rhythmic genes genome wide. This includes a 10 of the 12 genes encoding enzymes involved in carotenoid synthesis from the geranlygeranyl diphosphate (GGDP) precursor suggesting circadian control of abscisic acid biosynthesis (Covington et al., 2008).

Using a triple knockout mutant line, the PRR transcription factors were shown to regulate ABA biosynthesis (Fukushima et al., 2009). PRR9, 7 and 5 negatively regulate carotenoid biosynthesis and consequently ABA production, and the triple knockout exhibited increased ABA levels leading to developmental issues such as dwarfing.

Not only is the abundance of ABA under circadian control but so is the ability to perceive it. The putative ABA receptor ABAR (CHLH/GUN5) exists in a feedback loop with TOC1. ABAR induces expression of the circadian oscillator gene *TOC1* and in turn is then negatively regulated by TOC1 due to direct binding of

TOC1 to the *ABAR* promoter. ABAR expression is altered in *TOC1* mis-expression mutants with TOC1 RNAi plants producing a phase advance whilst TOC1 overexpresser plants have reduced ABAR abundance. As a result, ABA has been shown to acutely induce *TOC1* expression at time points corresponding to day, when TOC1 levels are low and thus ABAR levels are high, but not night when the opposite is true (Legnaioli et al., 2009; Pokhilko et al., 2013). Recently it has been shown that the transcription factor MYB96 is essential for the gated induction of *TOC1* by ABA, binding to the *TOC1* promoter and positively regulating its expression (Lee et al., 2016). Transcriptome analyses revealed that *TOC1*, when mis-expressed, also results in altered expression of genes involved in the ABA signalling pathway (Legnaioli et al., 2009; Castells et al., 2010). Overexpression of TOC1 (TOC1-OX) resulted in down-regulation of many of these genes and this mis-regulation of ABA related transcripts had an impact at the physiological level with TOC1-OX lines becoming more susceptible to drought stress whilst the TOC1-RNAi line was more resilient compared to the wild type (Legnaioli et al., 2009).

Transcriptomic experiments, attempting to quantify the scope of the downstream effects of ABA, found 221 genes that were up regulated more than 3-fold (Takahashi et al., 2004) whilst a separate study identified 308 ABA regulated genes from guard and mesophyll cells (Leonhardt et al., 2004). Both of these studies used microarrays which represented only part of the *Arabidopsis* genome meaning that many ABA regulated genes were likely missed. A more recent study which used a 26K 70-mer oligo-microarray chip that contained all of the predicted genes for the *Arabidopsis* genome (Lee et al., 2004), found that 1887 genes were regulated by ABA (Huang et al., 2007). Analysis of promoter motifs revealed that within this set of 1887 ABA responsive genes, the circadian related evening element (AAAATATCT) motif (bound by LHY/CCA1) was the second most prevalent (the first being the ABRE). This creates a strong case for the importance of the circadian regulation of ABA

responsive gene transcription.

1.3.1 Aims

Using *Arabidopsis thaliana* as a model organism, the work presented within this thesis aims to address the following questions:

1. Does ABA affect the function of the plant circadian clock, and what are the mechanisms?
2. Does osmotic stress affect the function of the clock in a manner similar to ABA?
3. Does the circadian clock impact on responses to ABA?
4. Does the circadian clock impact on responses to drought and osmotic stress?

It is hoped that this information will provide insight into the role of the circadian clock on the regulation of abiotic stress responses which may aid in the future novel approaches to improving abiotic stress tolerance in crop plants.

Chapter 2

Materials and Methods

Unless stated otherwise, all procedures requiring the use of kits, such as RNA extraction and cDNA synthesis, were carried out according to the suppliers standard protocols. Standard molecular biology techniques such as agarose gel electrophoresis were performed as described by Sambrook and Russell (1983).

2.1 Plant Material

2.1.1 Wild-type lines

All wild-type lines were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC) (Scholl, 2000) and subsequently bulked using plant growth facilities at the University of Warwick: Columbia (Col-0, ID: N1092) , Landsberg erecta (Ler-0, ID: NW20), and Wassilewskija (Ws-0, ID: N1602).

2.1.2 Circadian clock gene mis-expressing lines

The *lhy-1* mutant, which overexpresses the *LHY* transcript referred to hereafter as *LHY-OX*, was described by Schaffer et al. (1998). The LHY knockout mutant *lhy-11* was described by Mizoguchi et al. (2002). *pLHY1, pLHY2* and *pLHY3* are *lhy-11* lines carrying a transgenic copy of the *LHY* coding region under the control

of its own promoter. They also contain an *LHY*::*luc* construct in which the *LHY* promoter is fused to the coding sequence for *luciferase*. These were generated by Dr. Jae-Yean Kim. All of these lines are in the Landsberg ecotype (Ler).

The *TOC1-OX* line (which overexpresses the TOC1 transcript) and the TOC1-RNA interference line (which has reduced levels of *TOC1* expression and referred to hereafter as *TOC1-RNAi*) were described by Más et al. (2003). Both of these lines are in the Columbia (Col) ecotype.

The *CCA1-OX* line overexpresses the *CCA1* transcript and was described by Wang and Tobin (1998). The *cca1-1* knockout mutant was described by Green and Tobin (1999). These lines are in the Columbia (Col) and Wassilewskija (Ws) ecotypes respectively.

2.1.3 Luciferase reporter lines

The luciferase reporter lines, in which the endogenous promoter of the gene of interest has been fused with the coding sequence for *luciferase*, were previously described by the following: *LHY*::*luc* and *CCA1*::*luc* (Kim et al., 2003), *TOC1*::*luc* (Alabadi et al., 2001), *PRR7*::*luc* (Salome and McClung, 2005), *PRR9*::*luc* (Para et al., 2007), *GI*::*luc* (Onai et al., 2004) and *LUX*::*luc* (Huang et al., 2012).

LHY promoter mutant lines

These lines were generated and described by Spensley et al. (2009).

2.1.4 ABA biosynthesis mutants

The ABA biosynthesis mutant lines were obtained from (NASC) and subsequently bulked using plant growth facilities at the University of Warwick: *aba1-4* (Ler, ID: N3101), *aba1-3* (Ler, ID: N3100), *aba2-4* (Col, ID: N3835) and *aba3-1* (Col, ID:

N157).

2.2 Surface Sterilisation of *Arabidopsis thaliana* Seed

2.2.1 For immediate use

Up to 200 µl of seed was aliquoted in a 1.5 ml eppendorf and soaked in 1 ml of 50 % bleach/H20 containing 0.02 % tween for 5 minutes. The seed was then washed 4 times in sterile water, and left to stand in the final wash until ready to use.

2.2.2 For short-term storage

Seed was treated as above with an additional final wash of 1ml 70-100 % ethanol, with immediate and thorough drying on sterile filter paper before storage.

2.2.3 Vapour-phase sterilisation

Alternatively, for simultaneous sterilisation of multiple lines and long-term storage the method used was that of Clough (2004). 200 µl of each seed line in open 1.5 ml tubes were placed in a desiccator jar along with a beaker containing 100 ml bleach. 3 ml concentrated HCl was added to the bleach in order to trigger the formation of chlorine gas. The container was then sealed and left in the fume hood for 5 hours.

2.3 Standard Plant Growth Conditions

Unless otherwise stated, *Arabidopsis* plants were grown under the following conditions. Sterile seeds were plated onto Murashige and Skoog (MS) agar (4.3 g/L Murashige and Skoog basal salt mix (Sigma-Aldrich), pH 5.6, 1.5 % (w/v) agar). Stratification treatment was performed in constant darkness at 4 °C for 3 nights. Plates were then transferred to a Sanyo Versatile Environmental Test Chamber (MLR-350) set at 22 °C under 100 µmol m^{-2} s^{-1} white light, 12L 12D.

2.4 Transfer Experiments

For experiments requiring the transfer of seedlings, *Arabidopsis* seeds were sown onto sterile cellulose membranes (Cellodisc). At point of transfer the cellulose membranes were carefully lifted, carrying the seedlings, and placed onto fresh media.

2.5 Germination Assays

In order to ensure comparable dormancy levels, seed was harvested simultaneously from all genotypes tested. Using a 1 cm^2 grid template, seeds were placed individually onto MS0 media, into the centre of each grid square. This resulted in 144 seeds per plate (36 seeds per plant line, and each quadrant a different plant line). When used, an aliquot of (+)-2-*cis*-4-*trans*-Abscisic acid (ABA) (Sigma-Aldrich) in methanol was added to molten MS0 media before pouring plates. Alternatively, D-sorbitol (Sigma-Aldrich) was added to fresh MS0 media prior to autoclaving. 6 independent progenies from individual plants were tested for Ler, *lhy-11* and *LHY-OX* lines whilst only one was used for each of the *pLHY* lines. After stratification, plates were transferred to a Sanyo Versatile Environmental Test Chamber (MLR-350) set at 22 °C under 100 μmol m^{-2} s^{-1} constant white light. Germination was scored daily over the 10 days using an Olympus SZX9 microscope with DF PL ZX-3 lens to detect radical emergence.

2.6 Luciferase Assays

Sterile *Arabidopsis* seeds were plated onto MS0 media in a 96-deep-well plate (Appleton Woods), with approximately 15 seeds per well. In order to prevent drying out of the media over the following growth and imaging periods, the outer rows and columns of wells were left empty and filled with sterile water. Plates were then sealed with optically transparent ThermalSeal RT^{TM}(Alpha Laboratories) and a

19G microlance^{TM}(Becton, Dickinson and Company) was used to create an ventilation hole for each well. After 7 days of growth, plates were sprayed with 5 μM Luciferin in 0.01 % (v/v) Triton R X-100 and returned to the incubator. On day 8, plates were transferred to an imaging chamber fitted with white LED lighting (Panasonic) and a photon counting camera where image acquisition commenced. During the first 24 hours in the imaging chamber the light regimen was set to match that of the growth environment, 12L 12D, 22 °C. This was changed to L/L (constant light) over the remaining imaging period to capture free-running circadian rhythms. Luminescence was monitored by digital imaging using either a Peltier cooled camera (ORCAII c4742-98 CCD); (Hamamatsu Ltd., Welwyn Garden City, UK) or a liquid nitrogen cooled camera (TEK 512x512DB DDC with an ST138 controller); (Princeton Instruments Inc., Trenton, New Jersey). Automated imaging protocols (see Figure 2.1), including control of illumination via the LED arrays, were created and run using the MetaMorph^{TM}software package (Molecular Devices Ltd., Wokingham, UK). Images were acquired with a 20 minute exposure at 2 hour intervals for 6-7 consecutive days, the first day under 12L 12D and the remaining days in L/L. Numerical data for the luminescence intensity were extracted from 16-bit image files using MetaMorph v7.7.3.0. In order to correct for noise, background intensities were measured for each image from regions containing no plants. This value was then subtracted from each data point per image across the time course. Temporal expression data was normalised to the mean expression observed in the constant light period. The mean and standard error of the mean were calculated from the background corrected data for each time point across the time course. Differences in mean *luciferase* expression between control and experimental conditions or control and mutated plant lines were assessed using two-tailed Student's T-tests with p-values less than 0.05 being considered significant.

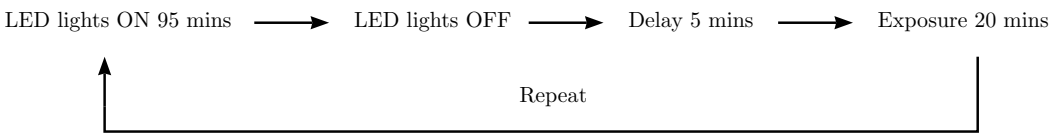

Figure 2.1 – Luciferase imaging routine. An image is acquired every 2 hours. To avoid capturing any delayed chlorophyll fluorescence signal, LED lights are switched off, followed by a 5 minute delay before the camera begins to acquire the image. A 20 minute exposure then occurs before the LED lights are switched back on. The loop repeats over the duration of the imaging experiment. This automated imaging protocol was implemented using the MetaMorph^{TM}software package.

2.7 Leaf Movement Assays

Arabidopsis seeds were plated onto MS0 media in a 1 cm^2 grid formation. After 10 days of growth, seedlings were transferred to fresh media containing 100mM D-sorbitol (Sigma-Aldrich) or sprayed with 25 µM ABA. Individual seedlings were then cut out of the agar using a scalpel, removing a 2 cm x 2 cm agar square with the seedling in the centre. These were then placed on the shelves of a gridded plate (ThermoFisher Scientific) sealing the plate with parafilm to limit moisture loss. Plates were aligned in an upright position in front of a TLC200 time-lapse camera (Brinno) with images acquired at 10 minute intervals over a 7 day period as described by Edwards and Millar (2007). The resulting .avi file was then analysed using ImageJ to detect the leaf movement within defined regions.

2.8 RNA Extraction & cDNA Synthesis

Arabidopsis seedlings were harvested into eppendorfs and flash frozen in liquid nitrogen. Two chilled 3 mm glass beads (Lenz) were added to each sample before loading into an MM300 Tissue Lyser (Retsch) for 1 minute at 30 Hz. Total RNA was extracted from the resulting powdered tissue using the Spectrum^{TM}Plant Total RNA kit (Sigma-Aldrich) and contaminating genomic DNA removed by treatment with DNaseI (Sigma-Aldrich). First-strand cDNA synthesis was performed using the M-MLV reverse transcriptase kit (Promega) which includes an RNase inhibitor

and random DNA hexamer primers.

2.9 Quantitative PCR (qPCR)

In order to determine transcript levels of coding sequences from RNA extractions quantitative PCR was performed using an Agilent Mx3005P detection system and the GoTaq^{TM}master mix reagent (Promega). Experiments conducted at the Bayer CropScience campus in Ghent Belgium were performed on a CFX384 system (Bio-Rad) using the Power SYBR^{TM}Green reagent (Applied Biosystems). Expression levels were calculated relative to the constitutively expressed gene *ACT2* (At3g18780) which had been shown to be stably expressed in response to ABA treatment via NanoString analysis (digital gene expression analysis, performed by Dr. Sally Adams) and in response to sorbitol treatment via meta-analysis of publicly available microarray data. The averaged stability ratio for *ACT2* when comparing expression in treated and untreated conditions was 0.997, confirming that neither ABA nor sorbitol treatment affected expression levels of *ACT2*. Furthermore, no significant time dependent differences in expression were detected over 24 hours. Each reaction was prepared in technical triplicate using 96 well plates for the Aglient system or 384 well plates for the CFX384 system using specifically designed primer sets (see Table 2.1). Differences between samples were assessed using paired Student's T-tests with p-values of less than 0.05 being considered significant.

Gene Name	ATG Code	Forward Primer (5' - 3')	Reverse Primer (5' - 3')
ACTIN 2	At3g18780	TCA GAT GCC CAG AAG TCT TGT TCC	CCG TAC AGA TCC TTC CTG ATA TCC
LHY	At1g01060	GCA AAT CTT CAA GCT ACA ACA GCAT	TAT CTG GAG AAA CGA ACG GTA ATC A
CCA1	At2g46830	AAA GGT GTA GCT ATG GGT CAA G	CGT GGA GGA GGA ATA GCT ATG T
TOC1	At5g61380	GCT GAG GTA CAT CAC ACG AGA CA	ACT TCG TCT TGC CTC GAC ATC ATT A
PRR9	At2g46790	GCG TGG GAG GTT CTA AAG GAG	GCG TGG GAG GTT CTA AAG GAG
PRR7	At5g02810	TGA TGG GAC ACT AGT TAG GGA TGA T	GGC TGG ATT ATA CCT TGA GAA AGC
GI	At1g22770	GCT GTC TTT CTC CGT TGT TTC AC	ACC TGT CTC CAT CCT TGT TGC
LUX	At3g46640	TGG CGG TAG CAG CGG TAA	TCA TCT GTT GCG TTC CAT ACG
ELF3	At2g25930	CCA CCT CCT GGT AAT GGC TAC TTC	ATT GGG TTG TTG TTG TTG TTG TTG T
RD29A	At5g52310	GGT GGT GGT GCG ACG GAG GAG GTG	TCT GCA CCG GAA CAA CAG TGG AGC C
LEA	At2g21490	ACT GGG GAA GCT ATG GGA AC	AGT CGT TTG TGC AGC TTG AG
LEA7	At1g52690	ACT GGG GAA GCT ATG GGA AC	AGT CGT TTG TGC AGC TTG AG
LTI30	At3g50970	AAG CTT CCC GGT GGT CAT C	GCG ACT CAA TGA AAG AAA GCC

Table 2.1 – Primers designed for qPCR

2.10 Western Blotting

Arabidopsis seedlings were harvested, flash frozen in liquid nitrogen and then ground to a powder using a pestle and mortar. 500 µl of Radioimmunoprecipitation assay buffer (RIPA) was added to approximately 1 ml of powdered tissue and vortexed. Samples were centrifuged for 10 minutes at 13,400rpm at 4 °C. The supernatant was then removed and placed in a clean microfuge tube. Protein was quantified using a Bradford assay (Bio-Rad). A sample volume containing 35 µg of protein was then mixed with sample loading buffer in a 1:3 ratio before loading into a 7.5 % SDS-acrylamide gel. Proteins were transferred to a nitrocellulose membrane (Amersham) using the wet-transfer method. The membrane was stained with Ponceau S (0.1 % (w/v) Ponceau S in 5 % (w/v) acetic acid) for 5 minutes to confirm equal loading. The membrane was washed with TBST before blocking with 7 % milk (Marvel PBS) in TBST for 1 hour at room temperature. LHY serum antibody was added to the blocking solution at a 1:500 dilution in a final volume of 14ml and left to incubate with the membrane overnight at 4 °C on a gel-rocker. The membrane was then washed with TBST for 3 x 5 minutes. Donkey anti-Rabbit secondary antibody (Abcam) was then added to blocking solution at a 1:2000 dilution in a final volume of 14 ml and left to incubate with the membrane at room temperature for 1 hour on a gel-rocker. The membrane was then washed with TBST for 4 x 10 minutes. Exposure was performed using 300 µl of activated ECL reagent (Amersham) per membrane and chemiluminescent film (FujiFilm). Quantification was then performed using ImageJ allowing comparison of the band intensity.

2.11 Drought-shock Treatment

Multiple *Arabidopsis* seeds were sown onto soil (3:1, compost:vermiculite) in 24 well plastic trays. The position of the different genotypes was randomised within each experimental block. Each experimental block contained 2 trays, one for the control

condition and the other for the drought stress condition. 12 experimental blocks were used with each genotype being represented twice per tray, this gave a total of 24 biological replicates per genotype. Cold stratification treatment was performed in constant darkness at 4 °C for 3 nights in a cold-room. Trays were then moved to a growth chamber (ARALAB) under 16L 8D cycles (100 μmol m^{-2} s^{-1} white light), 70 % (RH) at 22 °C. After 7 days of growth the trays were thinned to leave only one seedling per well. During the first 14 days, all trays were watered every 3 days by soaking in water troughs until the topsoil appeared damp. During this period no photographs were taken. After 14 days, drought condition trays were no longer watered, whilst control condition trays continued to receive water every 3 days. Daily aerial photographs were then taken for 2 weeks in order to quantify rosette surface area over time. Photographs were taken with a Nikon D3100 Camera and Nikon DX 18-55mm stock lens using a 30cm ruler for scale.

2.11.1 Measurement of Rosette Area

Numerical data were extracted from the photographs using imageJ. A stack of images over time was created for each tray using the `stack_sorter` plugin (Dougherty, 2005). Colour thresholding (using the `threshold_colour` plugin (Landini, 2014)) was performed to remove all non-green pixels. Images were then converted to 8-bit and then further converted to binary using the Huang preset algorithm which includes hole filling. A further binary conversion was done under default settings so as to produce clean black plant shapes on a white background that could then be easily selected using the wand tool, to measure particle size in cm^2.

2.12 Mild Drought or Salinity Treatments

These experiments were performed at the Bayer CropScience facility in Ghent, Belgium, using an automated platform (Phenopsis) as originally developed in the In-

stitut National de la Recherche Agronomique (INRA) (Granier et al., 2006). 14 randomised and independent experimental blocks were designed giving two biological replicates per line per treatment on each block. Locally sourced outdoor soil was sieved to remove any large debris that would cause uneven water distribution in pots, then mechanically mixed with indoor soil (DCM sowing and cutting soil) at a 3:2 (v:v) ratio. The loose soil was moistened slightly whilst mixing by spraying a fertiliser solution into the machine in order to make it more compact and remove excess air. 504 numbered and perforated pots were filled with 190-200 g of the soil mixture and weighed and recorded using an ENTRIS 2202-1S digital scale (Sartorius). Soil samples were taken at the start, middle and end of a soil batch, soaked with water and weighed before being placed in an oven at 105 °C in order to calculate the maximum soil water capacity (SWC). Pots were sprayed with fertiliser solution for 1-2 seconds from a pressurised dispenser to dampen the top-soil. *Arabidopsis* seeds were sown onto the top soil in a triangle formation about 1cm apart using a paintbrush. The perforated pots were then placed in a corresponding non-perforated pot and stratified for 7 days in a cold room at 4 °C. Pots were then loaded into the Phenopsis growth chamber according to the randomised experimental layout under a 12L 12D light regimen and 65 % relative humidity (RH). Daily watering was automated, each pot being weighed and watered to reach the desired target weight based on the starting pot weight and the target SWC %. For the following 14 day period all pots received normal watering with fertiliser solution, maintaining 50 % of the SWC. During this period seedlings were thinned so as to leave only one per pot and weeded to remove any non-*Arabidopsis* plant material which had entered from the outdoor soil. After 14 days, the non-perforated pots were removed (replaced again after 7 days) to accelerate moisture loss from the soil. Control pots received watering to maintain 50 % SWC whilst the drought condition received 20 %. Salt treated pots received 150mM NaCl in the fertiliser solution from a separate supply line at 50 % SWC. Controlled watering continued for the next 14 days until the end

of the experiment. Visible spectrum and infrared aerial photographs of each pot were acquired daily over days 14-28 of the experiment (see Figure 2.2). After the final day of imaging, fresh weights were measured using an ENTRIS124-1S digital scale (Sartorius). All plants were then placed in individual paper envelopes before being moved to an oven to dry for 5 days at 60 °C before measurement of dry weights.

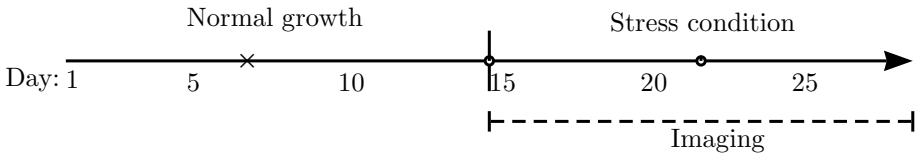

Figure 2.2 – Timeline of the phenopsis experiment after entering the growth chamber. Timing of events is indicated by the number of days. The first 14 days of growth are in control conditions allowing normal development. Thinning of seedlings and weeding out non-*Arabidopsis* material occurs on day 7 of growth, indicated by the 'x' mark. Conditions are then switched to the appropriate stress condition, indicated by the vertical line on day 14, which continues for the remaining 14 days. During this period, visible spectrum and infrared images are acquired daily. Open circles represent the removal and then replacement of non-perforated pots respectively.

2.12.1 Measurement of Rosette Surface Area and Analysis of Rosette Colour

Visible spectrum images were analysed using a Bayer developed pipeline in LemnaGrid which is part of the LemnaTec software package. 1. LAB colour space channels were removed except Green-Magenta resulting in grey scale images that were thresholded and hole-filled. 2. These images were used as masks to remove artefacts (such as moss) from the original images and create a binary image. 3. Small particles were removed and holes were filled a second time. In order to quantify the images, plant outlines were created from the binary image to create a region of interest (ROI). Using this ROI the rosette surface area was calculated by taking the total pixel count and then scaled using the number of pixels over a known distance. Colour analysis was performed by placing the ROI boundary over the original unprocessed

image and extracting averaged RGB values.

2.12.2 Infrared Thermography

Infrared images were analysed using a Bayer developed pipeline in LemnaGrid. This entailed: 1. The binary mask from the visible image was used to outline the ROI. 2. Colour classes were then defined based on the IR camera specifications allowing correlation between colour and temperature. 3. Colour class information was obtained for the ROI.

2.13 Phenotyping of Plants Treated with ABA or Sorbitol *in vitro*

These experiments were performed at the Bayer CropScience facility in Ghent, Belgium. Large tissue culture plates (150 x 25 mm) with an etched grid that divided the plate into 32 squares were used and a single seed was placed in each square (see Figure 2.3). The `rosettR` package for R was used to determine the layout of the plates and the randomisation of experimental blocks (Redestig, 2016; Tomé et al., 2016). Each plate was divided into 4 quadrants, each containing a separate genotype. Each of the 8 genotypes was tested under 5 conditions (MS0, 2 μM ABA, 10 μM, 150 mM sorbitol, 300 mM sorbitol). With 12 replicates (96 in total per genotype and condition) this resulted in a total of 120 plates. Working in a sterile laminar flow hood sterile *Arabidopsis* seeds were sown onto nylon membranes (Sefar) on MS0 media according to the layout file and stratified for 3 nights at 4 °C before growing under 12L 12D at 22 °C. In order to allow calculation of the relative growth rate prior to stress treatments plates were photographed after 7 and 10 days by placing onto a podium containing a built in light source underneath the stage. The nylon membranes containing the seedlings were then transferred to new plates containing either ABA or sorbitol and photographed daily for the remaining 8 days of the ex-

periment. Rosette area was then analysed in `rosettR`, which allowed image scaling, re-orientation of mis-aligned plates, identification of individual seedlings within the plate grid and the ability to track them across the course of the experiment.

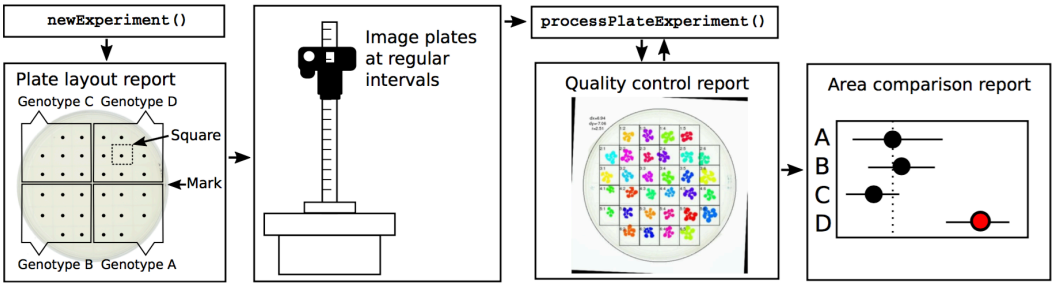

Figure 2.3 – `rosettR` protocol overview. The rosettR package for R was used to determine the layout of the plates and the randomisation of experimental blocks. Each plate was divided into 4 quadrants, each containing a separate genotype. After 7 days of growth the first photographs were taken daily using a back-lit imaging podium. Photographs were then taken daily between days 10-18 of growth. Images were then analysed in `rosettR`, which allowed image scaling, re-orientation of mis-aligned plates, identification of individual seedlings within the plate grid and the ability to track them across the course of the experiment. Figure reproduced from (Tomé et al., 2016).

2.14 ABA Quantification by Mass Spectrometry

Plants were grown as described in section 2.11. In order to induce drought stress watering was stopped after 14 days. After a further 10 days rosette samples were harvested and flash frozen. Samples were then homogenised by adding two chilled 3 mm glass beads (Lenz) to each sample before loading into an MM300 Tissue Lyser (Retsch) for 1 minute at 30 Hz. 10 mg of tissue was then weighed out using a digital scale and placed in a fresh eppendorf tube. 400 µl of extraction buffer, (Fisher Scientific Optima^{TM}LC/MS grade components: 10 % MeOH, 1 % acetic acid (v/v), containing the labelled ABA standard, Abscisic acid-d6 (Chiron), was added to each sample and placed on ice for 30 minutes. Samples were then centrifuged at 13,000 rpm at 4 °C for 10 minutes. The supernatant was removed and placed in a new eppendorf tube. 400 µl of extraction buffer without the labelled ABA standard was

then added to each pellet, re-suspended and left on ice for 30 minutes. Samples were centrifuged again at 13,0000rpm for 10 minutes at 4 °C. The supernatant was removed and combined with the previous supernatant which resulted in a total volume of 800 µl. Extraction blanks (no plant tissue) and solvent blanks (no plant tissue or labelled standard) were also created as controls. 15 µl of each Sample was then loaded onto a Xevo TQ-S UPLC-MS/MS system (Waters) and analysed by HPLC-electrospray ionisation/MS-MS. Chromatographic separation was performed using a C18 100 mm x 2.0 mm column (Acquity), at 35 °C. Machine optimisation, collision energies, solvent gradients and other operation details were performed as described in Forcat et al. (2008). Samples were analysed in technical triplicate with a solvent blank run between each sample to prevent carry-over of compounds. Extraction blanks were run systematically throughout the sample list to ensure there was no contamination between samples. Data was acquired and analysed using the MassLynx suite (Waters).

2.15 Bioinformatic Methods

2.15.1 Arabidopsis *cis*-Element Database

Circadian clock gene promoters were scanned for previously described *cis*-regulatory elements using the Arabidopsis cis-regulatory element database (AtcisDB) (Yilmaz et al., 2011), accessible at:

`http://arabidopsis.med.ohio-state.edu/AtcisDB/`

2.15.2 Creation of ABA Responsive Gene Lists

A list of ABA regulated genes was compiled by retrieving those which showed 2-fold up or downregulation from the Transcriptional Regulation By ABA Signalling (TRABAS) database (Choudhury and Lahiri, 2008), accessible at:

`http://www.bioinformatics.org/trabas/`

This list was then compared with data from 5 publicly available experimental data sets exploring the effect of ABA (see Table 2.2). These were accessed through the Genevestigator platform (Zimmermann et al., 2004; Hruz et al., 2008), accessible at: `https://genevestigator.com`

Or individually at the following:

AT-00420 : `http://www.ebi.ac.uk/arrayexpress/experiments/E-MEXP-2378/`

AT-00420 : `http://www.ebi.ac.uk/arrayexpress/experiments/E-MEXP-2378/`

AT-00433 : `https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE19520`

AT-00541 : `https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE28800`

AT-00637 : `http://www.ebi.ac.uk/arrayexpress/experiments/E-MEXP-3713/`

Genevestigator ID	Experimental Design	Reference
AT-00420	Plant samples of Col-0 grown for 2 weeks on MS agar medium with 3 % sucrose (16h light / 8h dark cycles, 22 °C) and then treated with 100 µM ABA (abscisic acid) for 1h.	(Mizoguchi et al., 2010)
AT-00420	Plant samples of Col-0 grown for 2 weeks on MS agar medium with 3 % sucrose (16h light / 8h dark cycles, 22 °C) and then treated with 100 µM ABA (abscisic acid) for 4h.	(Mizoguchi et al., 2010)
AT-00433	Excised leaf samples of 5 weeks old Arabidopsis thaliana Col-0 wild-type plants, treated with 50 mM ABA for 3h.	(Pandey et al., 2010)
AT-00541	Seedling samples Col-0 grown for 12 days on plates, then transferred into a solution containing 10 µM ABA (abscisic acid) for 6h.	(Kim et al., 2011)
AT-00637	Whole plant samples of Col grown for 2 weeks on solid germination medium (16h light / 8h dark, 22 °C), then placed into 10ml of water overnight, and then transferred into 10ml of 50 µM ABA (abscisic acid) solution for 90min.	(Umezawa et al., 2013)

Table 2.2 – Publicly available micro-array data sets used in the creation of ABA responsive gene lists

Chapter 3

Effect of Abscisic Acid on Circadian Clock Genes

3.1 Introduction

ABA was found to induce expression of the core clock gene *TOC1* (Legnaioli et al., 2009). This chapter describes efforts to test whether other core circadian clock genes are responsive to exogenous ABA application. We performed bioinformatic analysis which revealed that other circadian clock genes could be affected by ABA. This was followed up by analysis of mRNA expression levels of core clock genes after ABA application at 3 hour time intervals over 24 hours.

3.2 Results

3.2.1 All of the core circadian clock genes contain an ABRE or ABREL motif in their promoter sequence

As Legnaioli et al. (2009) has already demonstrated an affect of ABA on *TOC1* expression we wanted to quickly gain an insight as to whether other circadian clock genes may be responsive to ABA in *Arabidopsis*. We performed promoter anal-

yses to look for the presence of known ABA related motifs, the ABA-responsive element (ABRE) (Choi et al., 2000) and the ABRE-like (ABREL) (Shinozaki and Yamaguchi-Shinozaki, 2000). Promoter sequences of circadian clock genes were then analysed for the presence or absence of these motifs using the Arabidopsis cis-regulatory element database (AtcisDB) (Yilmaz et al., 2011) (see table 3.1).

This revealed that all of the core circadian clock genes contain at least one ABRE or ABREL motif and may, therefore, respond to ABA. *ELF3* contains the least number of ABA related motifs with a single ABREL present. *LHY*, *TOC1*, *PRR5* and *ELF4* have 2 whilst *LUX*, *CCA1* and *PRR9* have 3. *PRR7* had the most with 4.

Motif	Consensus Sequence	*LHY*	*CCA1*	*PRR9*	*PRR7*	*PRR5*	*TOC1*	*LUX*	*ELF4*	*ELF3*
ABRE	(C/T)ACGTGGC							1		
ABRE-like	(C/G/T)ACGTG(G/T)(A/C)	2	3	3	4	2	2	2	1	1

Table 3.1 –
A comparison of the ABRE (Choi et al., 2000) and ABREL (Shinozaki and Yamaguchi-Shinozaki, 2000) transcription factor binding motifs present in core circadian clock gene promoters. Consensus sequences and analysis were performed using the *Arabidopsis* Gene Regulatory Information Server (AGRIS) AtcisDB (database).

3.2.2 ABA causes induction of *LHY* and *CCA1* expression at dawn

In order to begin to test whether the expression clock associated genes is affected by ABA application, seedlings were grown under a 12L 12D light regimen for 7 days then transferred to constant light. ABA or vehicle (methanol) was sprayed onto the seedlings at either ZT24, 27, 30, 33, 36, 39, 42 or 45. Samples were harvested 3 hours after treatment and flash frozen in liquid nitrogen for RNA extraction, cDNA synthesis and analysis by qPCR. Gene expression levels were calculated relative to *ACTIN2*.

The well characterised ABA responsive gene *RESPONSIVE TO DESICCATION 29A (RD29A)* was chosen as a control to determine whether the ABA treatment was successful (Nakashima et al., 2006; Msanne et al., 2011). As expected, significantly ($p \leqslant 0.001$) elevated *RD29A* expression was observed in all ABA-treated samples independently of the time of day (see figure 3.1a).

Both *LHY* and *CCA1* transcripts were significantly induced in response to ABA at subjective dawn (ZT27 and ZT48) (see Figures 3.1b and c). Induction was also observed for both genes at time points corresponding to mid-day and early night (ZT30, 33, 36, 39 and 42) although the intensity of the response was much smaller.

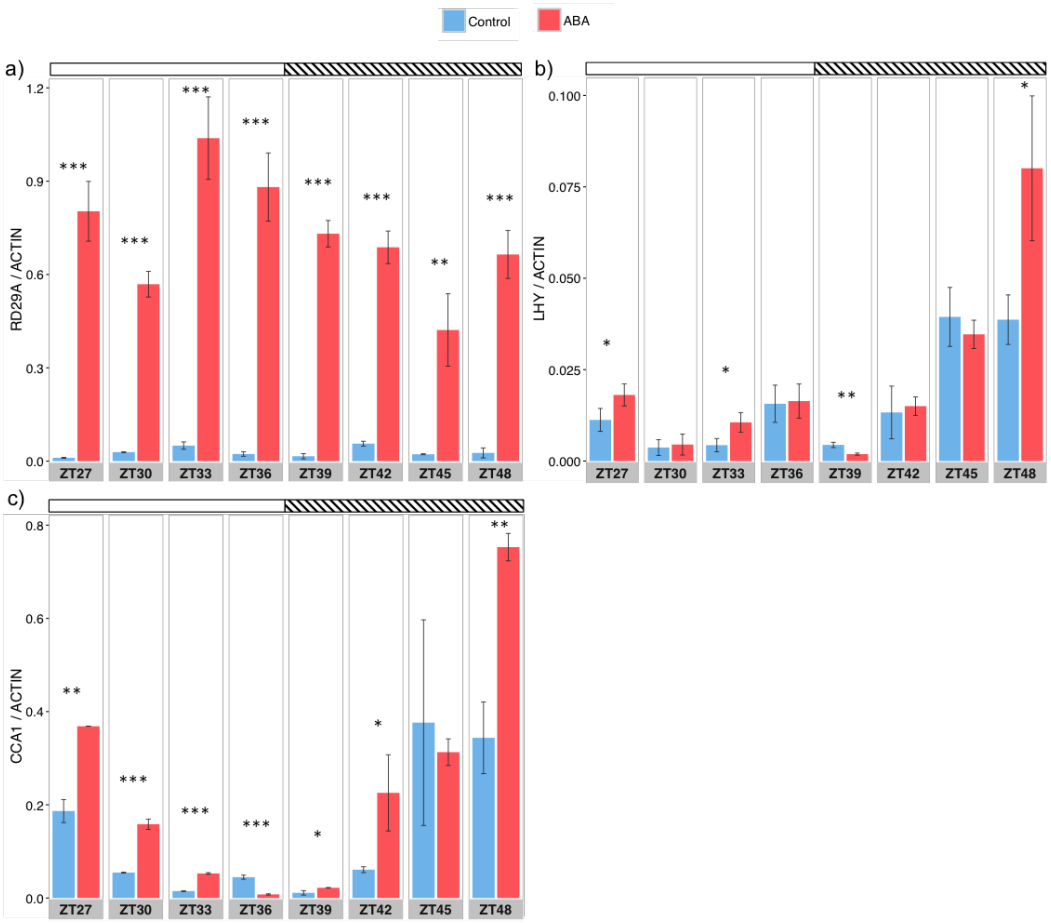

Figure 3.1 – ABA induces *LHY* and *CCA1* expression when applied at dawn. *Arabidopsis* seedlings (Ler) were grown on MS0 for 7 days at 22°C and entrained to a 12L 12D lighting regimen before being transferred to constant light. A single application of ABA (25 μM) or vehicle (methanol) was sprayed onto plants at one of the following time points (ZT24, 27, 30, 33, 36, 39, 42, 45). Plants were harvested 3 hours after and flash frozen. **a)** *RD29A*, **b)** *LHY* and **c)** *CCA1* transcript levels were analysed by qPCR and quantified relative to *ACTIN2* expression. Open and hashed bars represent subjective day and night, respectively. Data represents the mean of technical triplicates with error bars showing standard deviation. Results were consistent across three independent biological replicates. Asterisks indicate p-values from T-tests comparing control and treated conditions (* $p \leqslant 0.05$; ** $p \leqslant 0.01$; *** $p \leqslant 0.001$).

As ecotype differences in response to heat stress have been reported (Barah et al., 2013), we tested whether the responses observed were ecotype-specific. Figure A.1 (see appendix) shows that *LHY* expression is also responsive to ABA in Columbia and that this induction is gated with heightened sensitivity at dawn. This implies that these responses are likely to be conserved amongst various wild type *Arabidop-*

sis ecotypes and not an isolated phenomenon of the Landsberg line.

To test whether ABA-induced changes in *LHY* and *CCA1* expression persisted in the long term, transgenic seedlings containing either an *LHY*::*luc* or *CCA1*::*luc* reporter construct were entrained to 12L 12D cycles for 7 days before imaging in constant light using a photon counting camera. Plants were sprayed with ABA at: ZT48, 51, 54, 57, 60, 63, 66 or 69 so as to cover a 24h period at 3 hour intervals.

Application of 25 μM ABA at subjective dawn (ZT48) 2 hours before the peak of *LHY* and *CCA1* expression caused a sharp and significant induction in the mean *luciferase* luminescence level in both the *LHY*::*luc* and *CCA1*::*luc* reporter lines ($p \leqslant 0.001$ and $p \leqslant 0.05$, respectively) (see Figures 3.2a and 3.3a). The response for both reporter lines was rapid with effects observable in under 1 hour post application. No significant difference was observed between the experimental and control groups at any of the other time points tested in either line.

These results indicate that the expression of both *LHY* and *CCA1* is induced transiently in response to ABA application at dawn but that there is no long term effect.

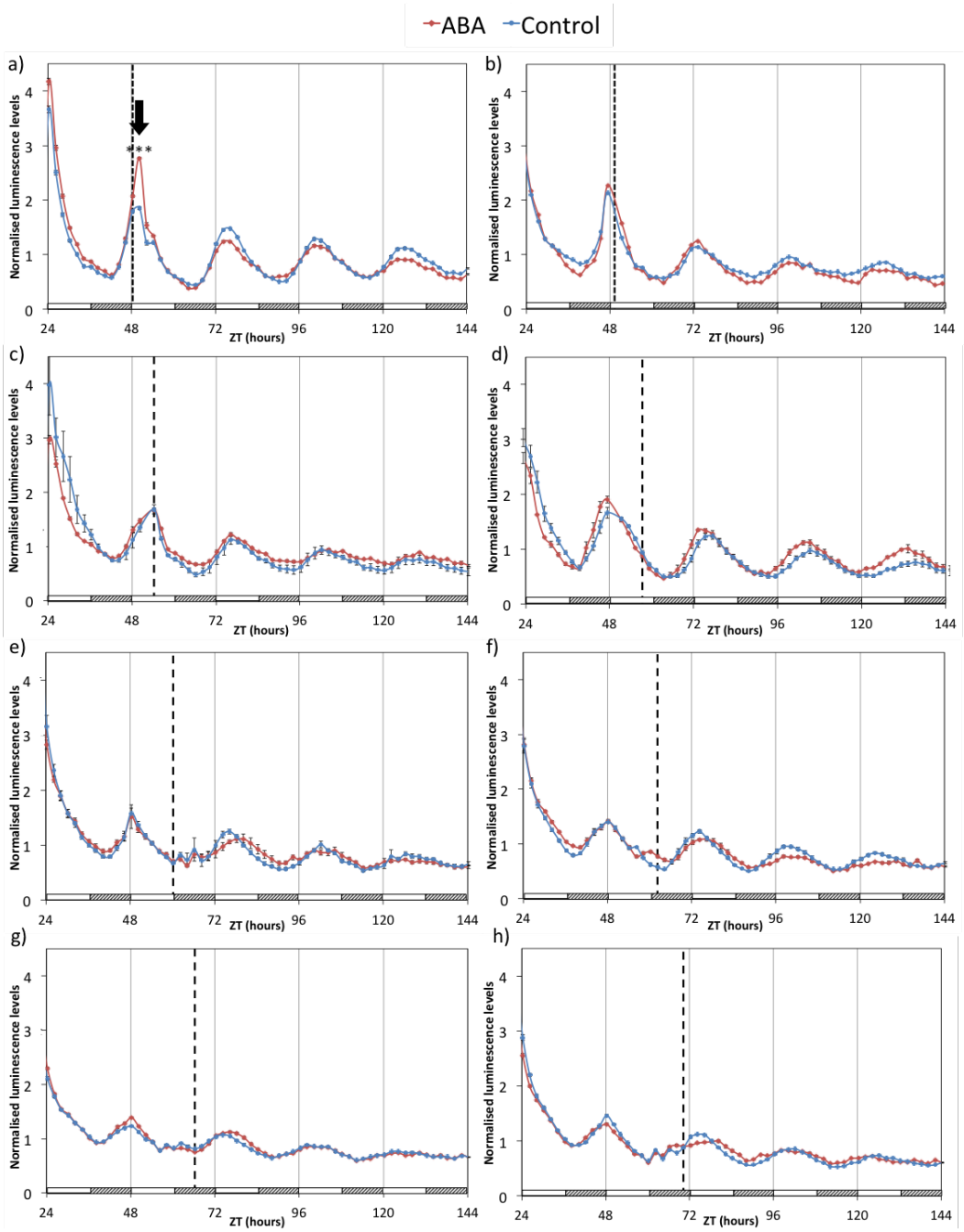

Figure 3.2 – ABA induces *LHY* expression when applied at dawn.
Arabidopsis seedlings (Ler) containing the *LHY*::*luc* reporter construct were grown on MS0 for 7 days at 22°C and entrained to a 12L 12D lighting regimen before being transferred to constant light. ABA (25 μM) or vehicle (methanol) was sprayed onto plants at either ZT48 (a), ZT51 (b), ZT54 (c), ZT57 (d), ZT60 (e), ZT63 (f), ZT66 (g) or ZT69 (h) as indicated by the dashed line. Data are means from at least 6 independent replicates, in at least two independent experiments. Temporal patterns of luminescence were normalised to the mean expression level in constant light. Error bars indicate the Standard Error of the Mean (SEM). Asterisks indicate p-values from T-tests comparing control and treated conditions (* $p \leqslant 0.05$; ** $p \leqslant 0.01$; *** $p \leqslant 0.001$).

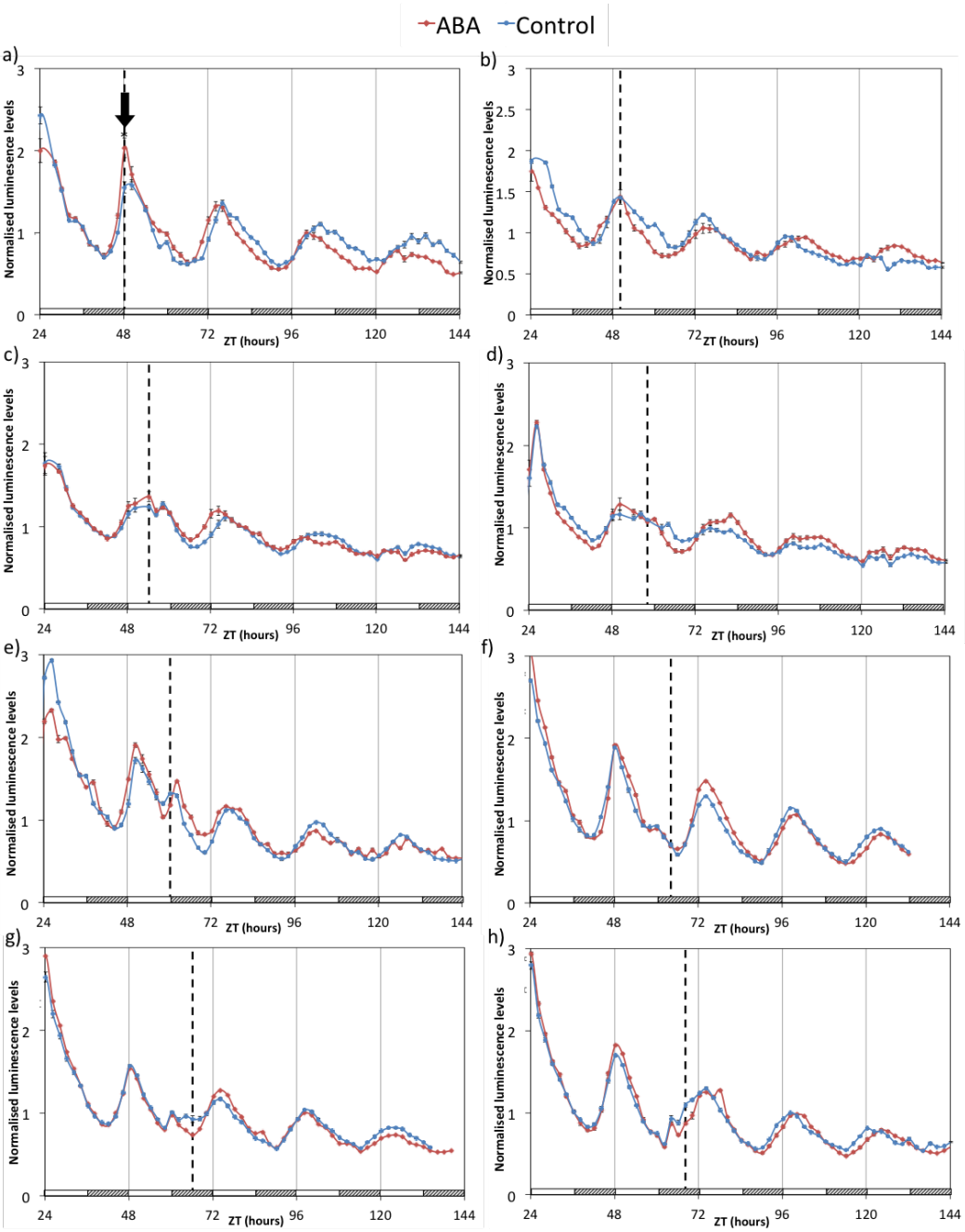

Figure 3.3 – ABA induces *CCA1* expression when applied at dawn.
Arabidopsis seedlings (Ler) containing the *CCA1*::*luc* reporter construct were grown on MS0 for 7 days at 22°C and entrained to a 12L 12D lighting regimen before being transferred to constant light. ABA (25 μM) or vehicle (methanol) was sprayed onto plants at either ZT48 (a), ZT51 (b), ZT54 (c), ZT57 (d), ZT60 (e), ZT63 (f), ZT66 (g) or ZT69 (h) as indicated by the dashed line. Data are means from at least 6 independent replicates, in at least two independent experiments. Temporal patterns of luminescence were normalised to the mean expression level in constant light. Error bars indicate the Standard Error of the Mean (SEM). Asterisks indicate p-values from T-tests comparing control and treated conditions (* $p \leqslant 0.05$; ** $p \leqslant 0.01$; *** $p \leqslant 0.001$).

Overall these findings support the results observed in the qPCR assay, showing that ABA acts to induce expression of *LHY* and *CCA1*. In addition they also confirm the narrow window of high sensitivity to ABA at dawn.

3.2.3 ABA induces *PRR9* and *PRR7* expression but reduces *TOC1* expression.

TIMING OF CAB EXPRESSION 1/PSEUDO-RESPONSE REGULATOR 1 (TOC1/PRR1) had previously been shown to be induced by ABA treatment in microarray experiments (Matsui et al., 2008) *luciferase* assays (Legnaioli et al., 2009), and qPCR experiments (Lee et al., 2016), all showing substantial induction around dusk.

However, we found that *TOC1* expression was slightly repressed by ABA at dusk (ZT39) and at night (ZT45). Slight, but significant induction was observed at ZT42. All other time points reported no significant response to ABA (see Figure 3.4a).

In contrast ABA induced *PRR7* expression during the subjective night with significant induction observed at ZT42, 45 and 48 and *PRR9* showed significant induction at all times with particularly large responses occurring at ZT27, 30 and 48 (see Figures 3.4b and c).

Together these results reveal that PRR9 and *PRR7* are induced in response to ABA and that the response is gated. However, we were unable to confirm the induction of *TOC1* expression during the night.

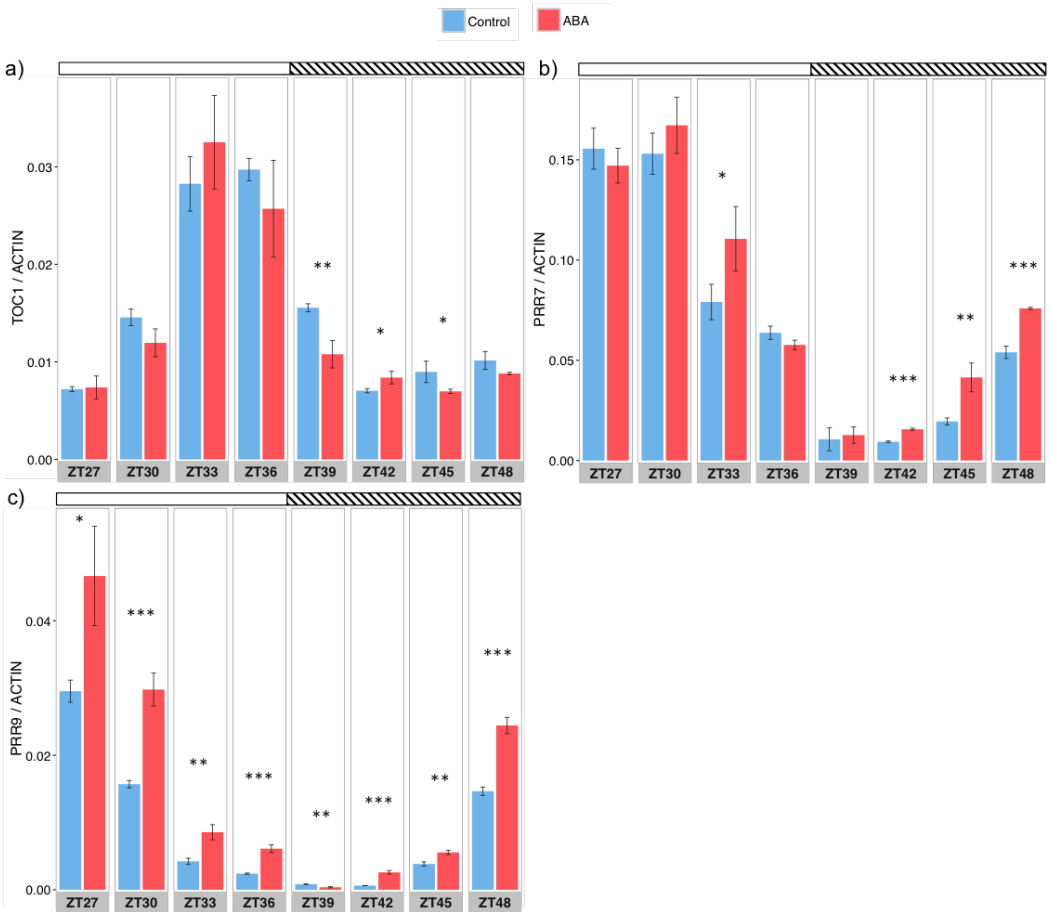

Figure 3.4 – ABA represses *TOC1* expression when applied at dusk. *Arabidopsis* seedlings (Ler) were grown on MS0 for 7 days at 22°C and entrained to a 12L 12D lighting regimen before being transferred to constant light. A single application of ABA (25 μM) or vehicle (methanol) was sprayed onto plants at one of the following time points (ZT24, 27, 30, 33, 36, 39, 42, 45), harvested 3 hours after and flash frozen. **a)** *TOC1*, **b)** *PRR7* and **c)** *PRR9* transcript levels were analysed by qPCR and quantified relative to *ACTIN2* expression. See figure 3.1c) for *RD29A* expression to show effective ABA treatment. Data represents the mean of technical triplicates with error bars showing standard deviation. Results were consistent across three independent biological replicates. Asterisks indicate p-values from T-tests comparing control and treated conditions (* $p \leqslant 0.05$; ** $p \leqslant 0.01$; *** $p \leqslant 0.001$).

In attempt to confirm these findings, the effects of ABA application on expression of *TOC1*::*luc*, *PRR7*::*luc* and *PRR9*::*luc* were assayed. *PRR9*::*luc* responded to ABA at most time points (see Figure 3.5). Applications before the endogenous peak of *PRR9* expression (ZT48, 51, 54, 57) resulted in rapid and significant induction. In contrast, ABA application after the peak (ZT60, 66 and 69) resulted in increased

amplitude of the next circadian peak. This may be due to reduced sensitivity of the *luciferase* technique in comparison to qPCR which was not able to resolve smaller changes at the trough of expression. The only time point at which significant induction was not observed at all was ZT63.

PRR7::*luc* and *TOC1*::*luc* were also tested but the lines failed to produce a clear signal and so were not able to produce informative data.

These results corroborate the qPCR findings, showing that *PRR9* is induced in response to ABA across the whole 24 hour period (with exception of ZT63).

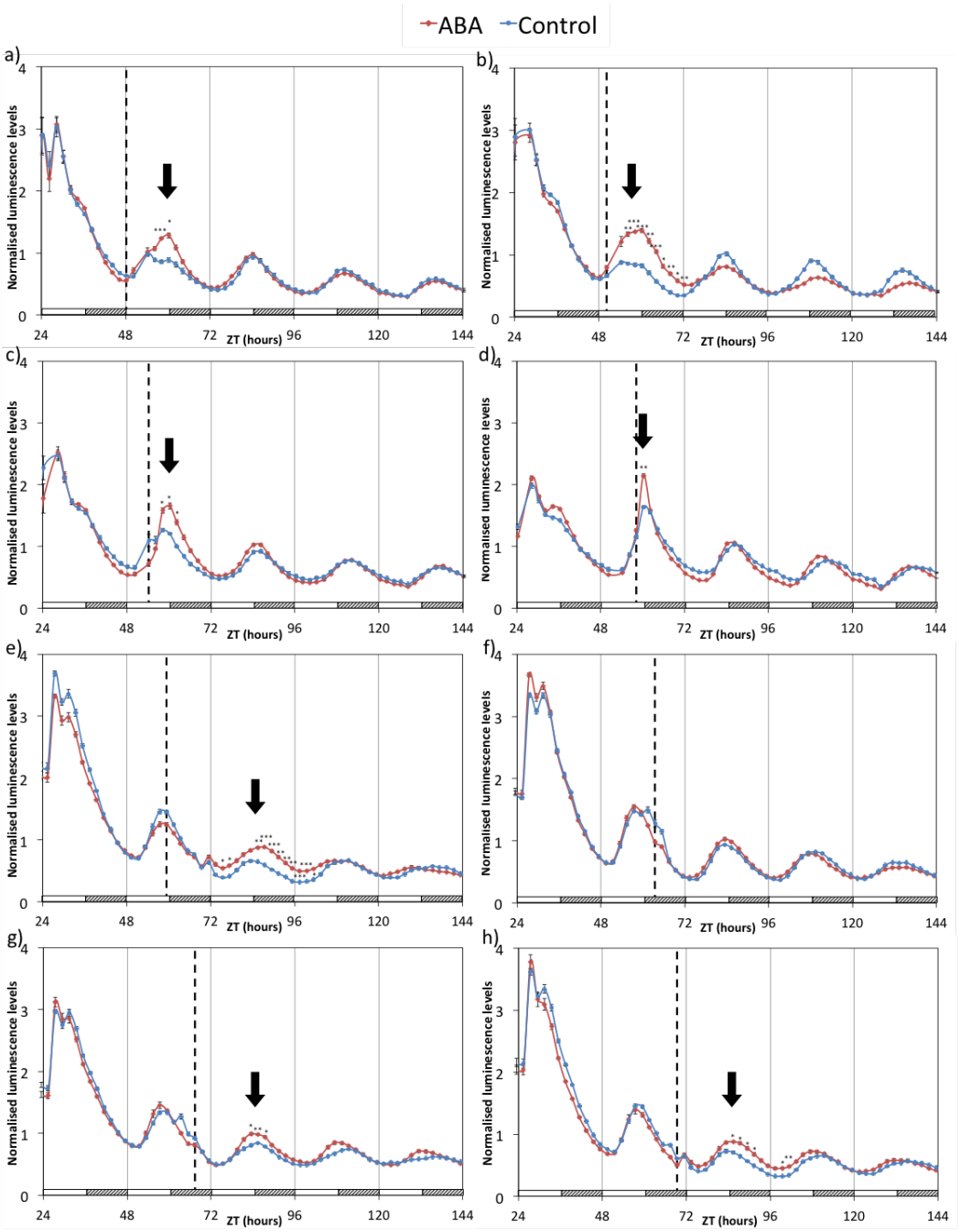

Figure 3.5 – ABA induces *PRR9* expression when applied at multiple time points over a 24 hour period. *Arabidopsis* seedlings (Ler) containing the *PRR9*::*luc* reporter construct were grown on MS0 for 7 days at 22°C and entrained to a 12L 12D lighting regimen before being transferred to constant light. ABA (25 μM) or vehicle (methanol) was sprayed onto plants at either ZT48 (a), ZT51 (b), ZT54 (c), ZT57 (d), ZT60 (e), ZT63 (f), ZT66 (g) or ZT69 (h). Data are means from at least 6 independent replicates with 15 seeds per well, in at least two independent experiments. Temporal patterns of luminescence were normalised to the mean expression level in constant light. Error bars indicate the Standard Error of the Mean (SEM). Asterisks indicate p-values from T-tests comparing control and treated conditions (* $p \leqslant 0.05$; ** $p \leqslant 0.01$; *** $p \leqslant 0.001$).

3.2.4 ABA does not significantly affect the expression of Evening Complex (EC) components *LUX* and *ELF3*

The Evening Complex (EC) is composed of three members, LUX ARRYTHMO (LUX), EARLY FLOWERING3 (ELF3) and EARLY FLOWERING4 (ELF4) and acts to negatively regulate *PRR9*, *PRR7*, *PRR5* and *TOC1* by binding directly to their promoters.

Although both genes contain ABRE or ABREL motifs in their promoters, *ELF3* was not significantly affected by ABA application at any time of the day (see Figures 3.6a and b). Alternately, *LUX* was affected at a single time point, being significantly reduced at ZT36. This implies that ABA does not strongly affect the function of the clock via an effect on the EC.

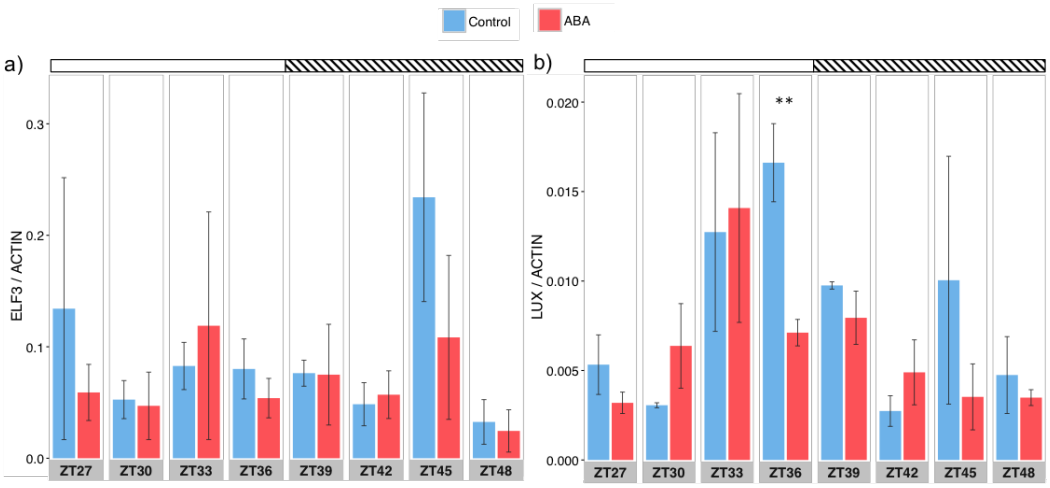

Figure 3.6 – ABA represses *LUX* expression when applied at ZT36 *Arabidopsis* seedlings (Ler) were grown on MS0 for 7 days at 22°C and entrained to a 12L 12D lighting regimen before being transferred to constant light. A single application of ABA (25 μM) or vehicle (methanol) was sprayed onto plants at one of the following time points (ZT24, 27, 30, 33, 36, 39, 42, 45), harvested 4 hours after and flash frozen. **a)** *LUX* and **b)** *ELF3* transcript levels were analysed by qPCR and quantified relative to *ACTIN2* expression. See figure 3.1c) for *RD29A* expression to show effective ABA treatment. Open and hashed bars represent subjective day and night, respectively. Data represents the mean of technical triplicates with error bars showing standard deviation. Results were consistent across three independent biological replicates. Asterisks indicate p-values from T-tests comparing control and treated conditions (* $p \leqslant 0.05$; ** $p \leqslant 0.01$; *** $p \leqslant 0.001$)

3.3 Discussion

We have shown that all of the core *Arabidopsis* circadian clock genes contain at least one ABRE or ABREL motif in their promoter sequence. Overall, the data presented in this chapter shows that the morning associated circadian clock genes, *LHY* and *CCA1* respond to ABA, and that this response is an acute induction that is gated to a small window around dawn. It would appear that ABA and ABA signalling have multiple entry points into the central clock architecture and therefore have the potential to alter clock function. As ABA is a hormone that is crucial for abiotic stress responses, this could be a mechanism through which stress signaling is mediated to the central oscillator in order to efficiently manage and adapt to stress conditions.

We have also shown that both *PRR9* and *PRR7* are induced in response to ABA, and that *PRR9* is responsive at all time points across the day. No significant effect of ABA was observed on the expression of the EC component *ELF3* although the lack of strong basal rhythmicity of expression in combination with the large standard deviations occurring at ZT27, 33 and 45 could potentially mask more nuanced responses. *LUX*, however, was responsive to ABA, repressing expression at ZT36 which corresponds with its peak of expression. This difference is interesting as both of these genes contain ABRE/ABREL motifs and demonstrates that the presence of an ABRE or ABREL motif is not sufficient for response to ABA and suggests that the context in which this motif occurs is important. However, we were unable to confirm induction of *TOC1*, which had been reported by Legnaioli et al. (2009). This may be because their growth medium included MS media supplemented with 3% sucrose. Previous qPCR experiments have also failed to show induction of *TOC1* in response to ABA on media without added sucrose (Hanano et al., 2006).

Prior to these experiments, *TOC1* was considered the most important clock gene in response to ABA, functioning in a nested feedback loop with the ABAR. ABA induces *TOC1* expression which in turn leads to negative regulation of the ABAR as *TOC1* binds to the *ABAR* promoter repressing transcription (Legnaioli et al., 2009). This led to the publication of a model which incorporates this loop and predicted that ABA would produce a slight dampening effect on *LHY* expression (Pokhilko et al., 2013) due to the negative effect TOC1 has on *LHY* (Gendron et al., 2012). However, treating *TOC1* as the only input for ABA signaling has led to an inaccurate prediction, at least in response to short term ABA responses, as we have shown that ABA induces LHY expression at dawn. Future work in updating this model to include our observed effects of ABA on *LHY*, *CCA1*, *PRR9* and *PRR7* would be useful in order to further understand the net effect of ABA on the circadian clock.

In conclusion we have shown that ABA affects clock gene expression and that this is a potential mechanism by which osmotic stress can affect the function of the clock.

Chapter 4

Effect of Osmotic Stress on Circadian Clock Genes

4.1 Introduction

The results described in Chapter 3 demonstrated that all core circadian clock genes contain at least one ABRE or ABREL motif and that ABA application can affect the expression of the core circadian clock genes *LHY*, *CCA1*, *PRR9* and *PRR7*. ABA is a key hormone in the response to abiotic stress and as described in Chapter 1 there have been a number of reports documenting the effect of abiotic stress on the circadian clock across a range of plant species. This chapter describes work aimed to test whether ABA signaling might contribute to the effect of drought and osmotic stress on the circadian clock.

4.1.1 Aims

The experimental aims were as follows:

(i) Investigate the effect of osmotic stress on circadian clock gene expression

(ii) Determine whether the ABA signaling pathway is necessary for the observed

effects of osmotic stress on the plant circadian clock

4.2 Results

4.2.1 Osmotic stress results in down regulation of circadian clock genes

As osmotic stress is known to elevate ABA (Creelman and Zeevaart, 1985) we tested whether osmotic stress would also affect circadian clock gene expression using *Arabidopsis* seedlings (Ler) containing *luciferase* reporter constructs (*LHY*::*luc*, *CCA1*::*luc*, *TOC1*::*luc*, *PRR9*::*luc* or *PRR7*::*luc*). The plants were imaged every 2 hours over 6 days. Seedlings were transferred to fresh media after the third day of imaging.

Transfer from the standard growth conditions (MS0) to media containing 100 mM sorbitol, resulted in dampened expression of all circadian clock genes within 24 hours of transfer (see Figure 4.1). Continual growth under osmotic stress resulted in persistently dampened oscillations and these were not rescued when plants were transferred back to medium without sorbitol.

These results indicate that the effect of osmotic stress on the amplitude of circadian clock genes occurs after approximately 24 hours of exposure. It also suggests that recovery of circadian oscillations requires a longer time span than 3 days for plants which have endured long term osmotic stress. Rhythms were not comparable to their un-stressed counterparts once transferred from osmotic stress to MS0 media.

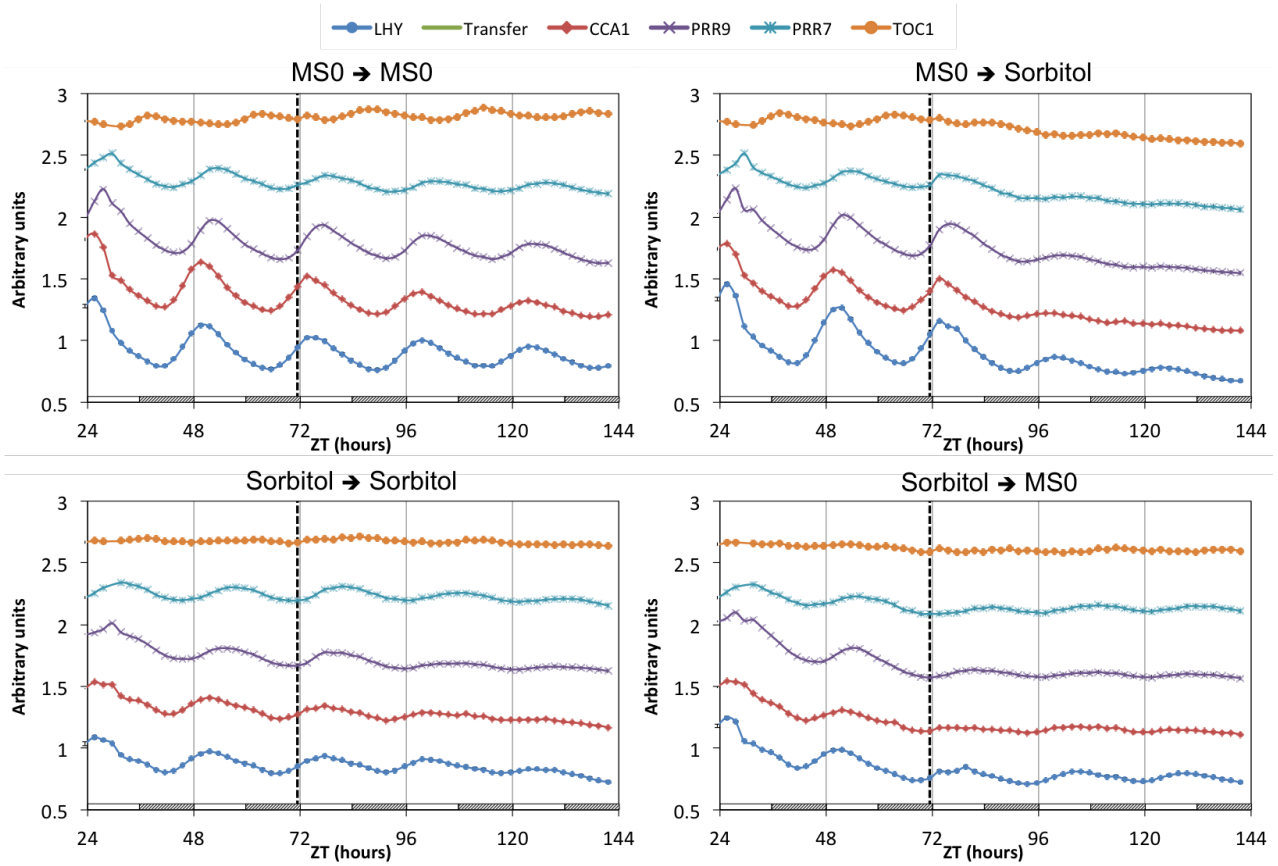

Figure 4.1 – Short term osmotic stress dampens *PRR9* and *PRR7* and *TOC1* expression. *Arabidopsis* seedlings (Ler) containing *luciferase* reporter constructs were grown on MS0 for 7 days at 22°C and entrained to a 12L 12D lighting regimen before being transferred to constant light. After 3 days of imaging seedlings were transferred to fresh media, either MS0 or MS0 containing 100 mM D-sorbitol. Imaging continued for a further 3 days. **a)** MS0 transferred to MS0, **b)** MS0 transferred to sorbitol, **c)** Sorbitol transferred to MS0, **d)** Sorbitol transferred to sorbitol. Open and hashed bars represent subjective day and night, respectively. Data are means from at least 6 independent replicates with 15 seeds per well, in at least three independent experiments. Y-axis is shown in arbitrary units as data has been separated vertically for representation on a single plot via addition. Temporal patterns of luminescence were normalised to the mean expression level in constant light. Error bars indicate the Standard Error of the Mean (SEM).

4.2.2 Long term osmotic stress affects *LHY*, *CCA1*, *TOC1*, *PRR9*, *PRR7* and *LUX* gene expression

The above effects of osmotic stress on the amplitude of circadian oscillations were confirmed by quantifying the endogenous levels of circadian clock gene transcripts. Seedlings were grown for 7 days on MS0 or MS0 containing 100 mM sorbitol at 22°C under a 12L 12D light regimen, which was changed to constant light on day 7. On day 8 samples were harvested at 3 hour intervals across a 24 hour period and flash frozen in liquid nitrogen for RNA extraction, cDNA synthesis and analysis by qPCR. Gene expression levels were calculated relative to *ACTIN2*.

RD29A was chosen as a control to determine whether the osmotic stress treatment was effective (see Figure 4.2a). As expected, significant ($p \leqslant 0.001$) induction was observed in stressed samples at all time points, with the exception of ZT 36 with significance of ($p \leqslant 0.01$).

In confirmation of the *luciferase* assay results above, both *LHY* and *CCA1* have a reduced amplitude of expression. Both genes are significantly repressed in the sorbitol condition when compared to the control at all time points tested except for one (see Figures 4.2b and c). The greatest differences are observed at time points corresponding to subjective dawn ZT24, 27, 30 and 45.

Long term osmotic stress caused significant ($p \leqslant 0.001$) induction of *TOC1* at dawn and mid day but repression during the evening and night (see figure 4.2c, d and e). *PRR7* showed mild but significant repression at mid day and dawn whilst *PRR9* showed slight but non-significant induction at ZT24 and 36. Given the mild effect of long term osmotic stress on *PRR9*, *PRR9* and *PRR7* expression we tested the affect on *LUX*, which showed strong repression in response to osmotic stress and its peak of expression (ZT33 and 36) was abolished (see Figure 4.2g).

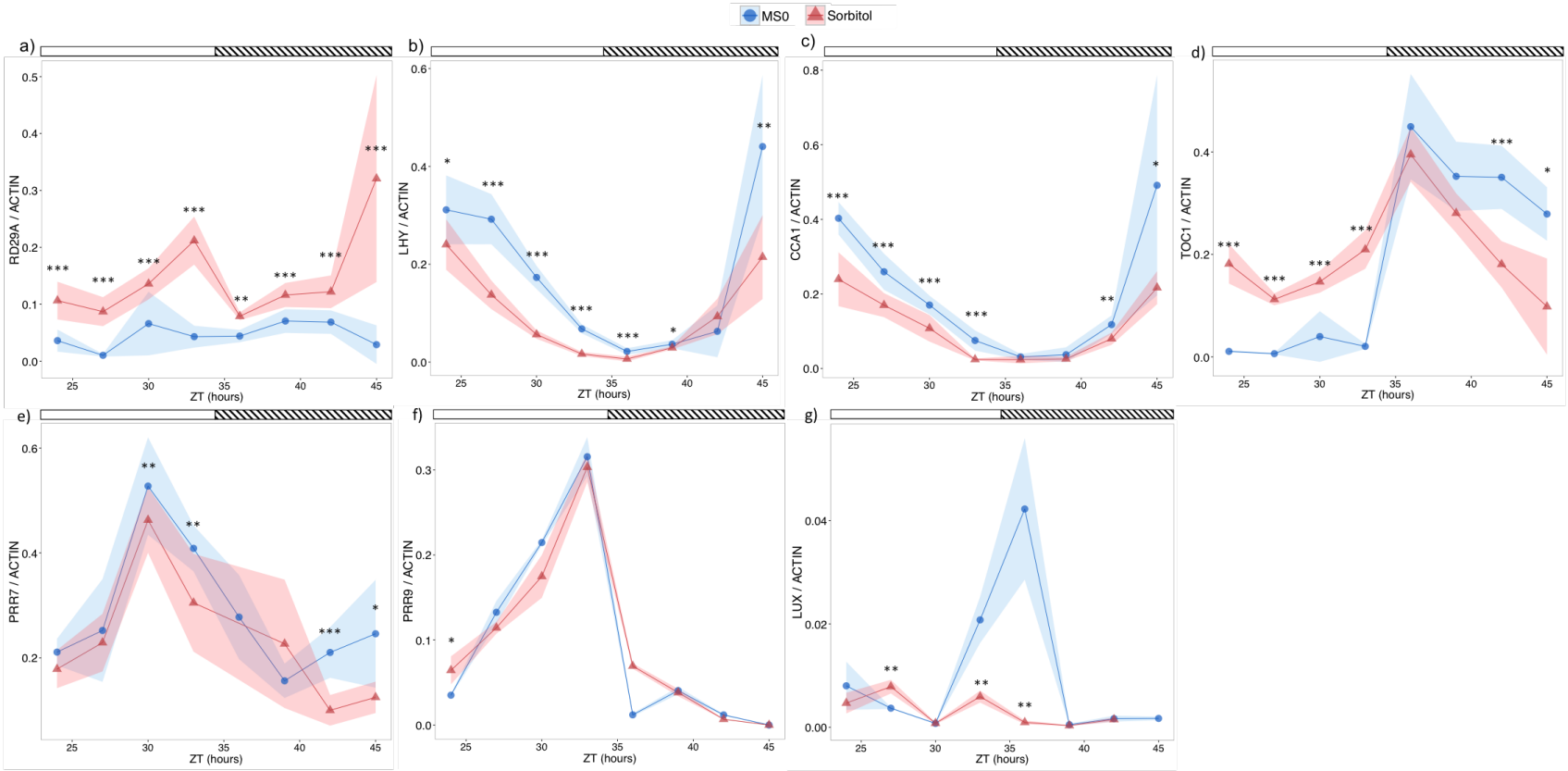

Figure 4.2 – Osmotic stress represses *LHY* expression at all times of day. *Arabidopsis* seedlings (Ler) were grown on MS0 or MS0 containing 100 mM sorbitol for 7 days at 22°C and entrained to a 12L 12D lighting regimen before being transferred to constant light. On day 8, samples were harvested at 3 hour intervals across a 24 hour time period and flash frozen. **a)** *RD29A*, **b)** *LHY*, **c)** *CCA1*, **d)** *TOC1*, **e)** *PRR7*, **f)** *PRR9* and **g)** *LUX* transcript levels were analysed by qPCR and quantified relative to *ACTIN2* expression. **a)** *RD29A* expression is included as a control to show effective osmotic stress treatment. Open and hashed bars represent subjective day and night, respectively. Data represents the mean of technical triplicates with ribbon thickness representing standard deviation. Results were consistent across three independent biological replicates. Asterisks indicate p-values from T-tests comparing control and treated conditions (* $p \leqslant 0.05$; ** $p \leqslant 0.01$; *** $p \leqslant 0.001$).

Overall these findings support the results observed in the *luciferase* assay, showing that long term osmotic stress acts to reduce the amplitude of *LHY*, *CCA1*, *TOC1*.

4.2.3 The effect of long term osmotic stress on *LHY* expression requires the G-box promoter motif

As ABFs are known to target G-box containing sequences (Choi et al., 2000) and our data has shown an effect of osmotic stress on *LHY* expression, we tested whether a functional G-box motif was necessary for this effect. We used a promoter mutant generated by Spensley et al. (2009), the -957 Wk Cl II *LHY*::*luc* reporter construct contains a full length promoter sequence but has a mutated G-box motif. Both 2-bp flanking sequences around the core hexamer were mutated (WT: acCACGTGtc Mutant: gtCACGTGac) (Spensley et al., 2009) as this had previously been shown to disrupt DNA binding protein complexes forming on G-box probes (Williams et al., 1992). This mutation resulted in reduced amplitude of circadian oscillations in both L/D and L/L cycles (Spensley et al., 2009).

Along with WT (Ws) this line was grown on standard growth medium (MS0) or medium containing 100 mM sorbitol, for 7 days at under a 12L 12D light regimen before being transferred to constant light for imaging with a CCD camera.

Loss of the G-box motif in the -957 Wk Cl II *LHY*::*luc* line resulted in abolishing the effect of osmotic stress entirely (see Figure 4.3). The amplitude of the luminescence rhythm was comparable in the presence and absence of sorbitol.

These results suggest that the G-box is a key binding motif in the *LHY* promoter for the integration of osmotic stress signal.

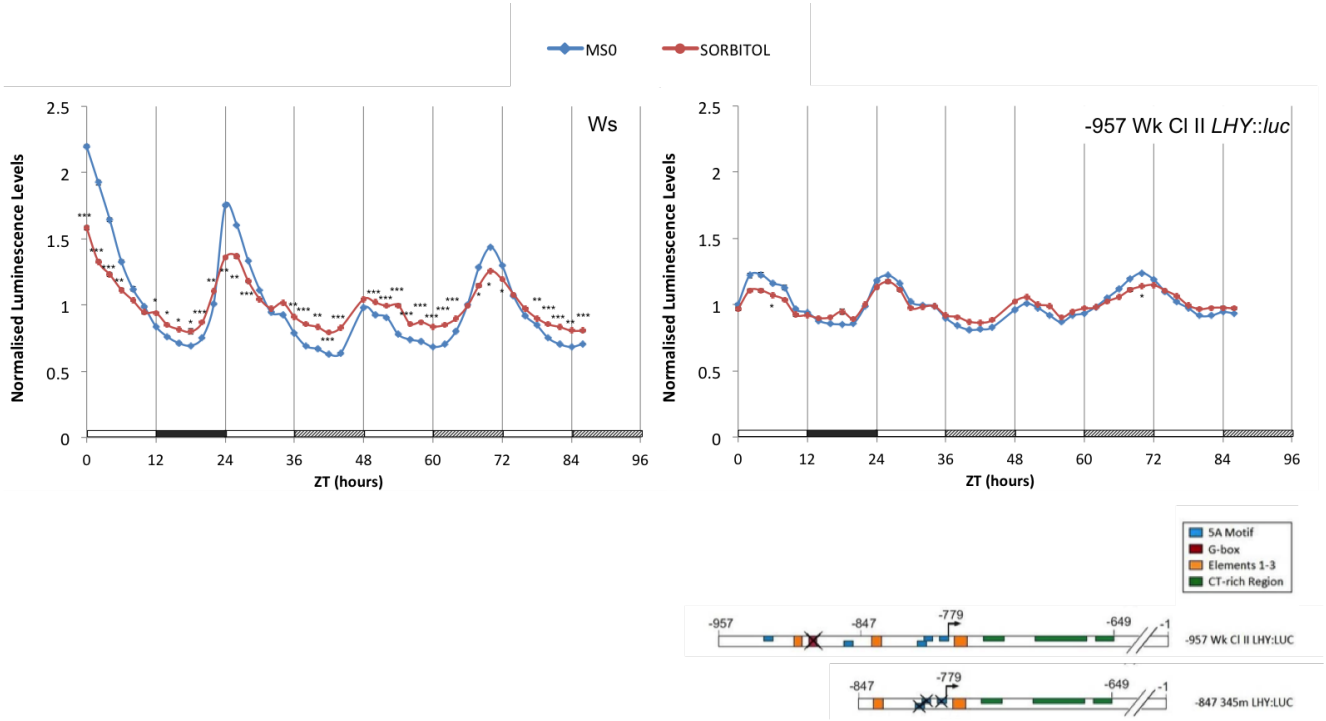

Figure 4.3 – The effect of osmotic stress on *LHY* expression requires the G-box motif in the *LHY* promoter. *Arabidopsis* seedlings containing *luciferase* reporter constructs for a series of promoter mutants were grown on MS0 or MS0 containing 100 mM D-sorbitol for 7 days at 22°C and entrained to a 12L 12D lighting regimen before being transferred to constant light. Data are means from at least 6 independent replicates with 15 seeds per well, in at least three independent experiments. Temporal patterns of luminescence were normalised to the mean expression level in constant light. Error bars indicate the Standard Error of the Mean (SEM). Asterisks indicate p-values from T-tests comparing control and treated conditions (* $p \leqslant 0.05$; ** $p \leqslant 0.01$; *** $p \leqslant 0.001$).

4.2.4 ABA is required for the effect of osmotic stress on leaf movement rhythms

As osmotic stress is known to induce elevated ABA levels (Zeevaart, 1980) and our data showed that ABA can affect the function of the clock, we tested whether a functional ABA signaling pathway was necessary for integrating the osmotic stress response with the circadian clock. To achieve this we used leaf movement, a circadian behaviour, as a proxy for circadian clock function to test whether ABA biosynthesis mutations abolished the effect of sorbitol stress on the clock.

ABA treatment and osmotic stress disrupted the rhythmic leaf movement in all WT ecotypes tested, reducing the amplitude or abolishing rhythmicity entirely (see Figure 4.4a, b and c).

In contrast, leaf movements in the ABA biosynthesis mutants *aba2* (required for the conversion of xanthoxin to abscisic-acid aldehyde (Schwartz et al., 1997*a*) and *aba3* (required for the function of aldehyde oxidase (AO) (Bittner et al., 2001) were similar in both the control and osmotic stress condition. This suggests that the inability to produce ABA in response to osmotic stress prevents the stress from affecting leaf movement rhythms (see figure 4.4d and e). This is further supported by the fact that when 25 µ mM ABA was applied exogenously rhythmic vertical leaf movement was disrupted similarly to that observed in the WT lines.

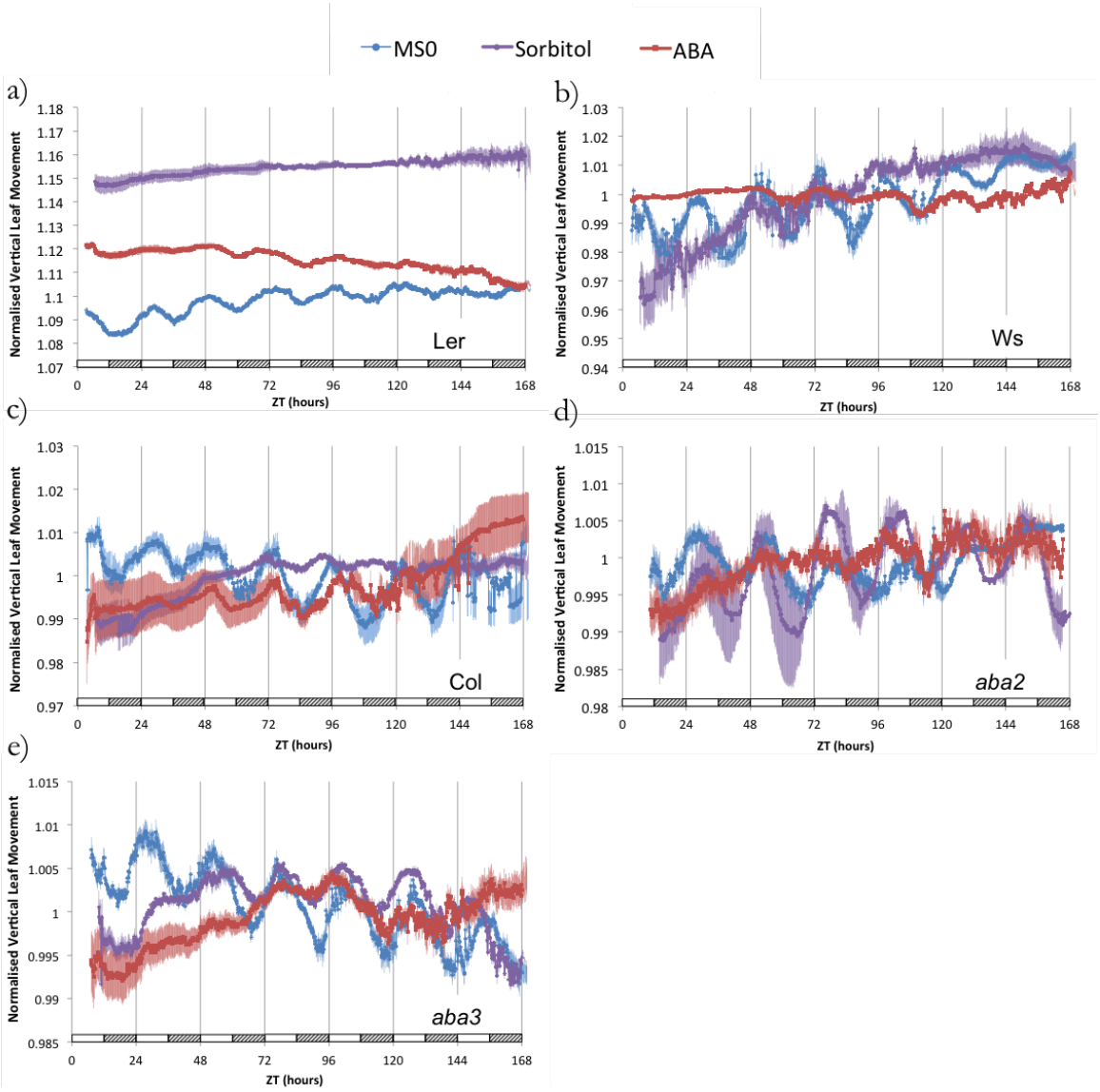

Figure 4.4 – ABA biosynthesis is required for osmotic stress to affect the circadian clock. Seedlings were grown on MS0 for 14 days at 22°C and entrained to a 12L 12D lighting regimen. They were then transferred to fresh MS0, MS0 containing 100 mM sorbitol or sprayed with 25 µM ABA and then individually placed into an upright gridded plate for time-lapse imaging; images were acquired at 10 minutes intervals over 7 days. (a) Landsberg, (b) Wassilewskija, (c) Columbia, (d) *aba2* (Col) and (e) *aba3* (Col). Vertical leaf movement was calculated using the image analysis software ImageJ. Open and hashed bars represent subjective day and night, respectively. Data are average-normalised. Error bars indicate the Standard Error of the mean (SEM).

4.3 Discussion

As ABA is upregulated in response abiotic stress, we hypothesised that osmotic stress may result in similar effects on circadian clock gene expression as had been seen in response to exogenous application of ABA. For example, acute induction of *LHY* and *CCA1* in the morning. We observed a reduction in levels for all clock genes, which contrasts with induction of these genes in response to ABA treatment. One explanation for this discrepancy may be that these two responses are occurring on two different timescales. Chapter 3 described acute responses to ABA, but these may be transient. The damping of the clock under long-term osmotic stress may be a secondary effect. Length of stress exposure has been shown to produce different transcriptional responses in core clock genes. In response to cold stress across a 14 day time course the initial 4 - 20 hours resulted in induction of most core circadian clock genes after which expression amplitude then reduced, excluding *LUX* which maintained high amplitude of expression (Bieniawska et al., 2008). Different transcriptional responses have also been observed in response to different stress severities with increased severity resulting in damping of clock gene expression. Mild drought stress of 30 % gravimetric humidity (GH) (70 % being typical field conditions) in soybean was not sufficient to result in reduction of expression amplitude. A stronger stress of 15 % GH reduced expression of evening expressed circadian genes including the *LUX* homolog (Marcolino-Gomes et al., 2014). Length of stress exposure may be a factor in, and contribute to, the overall stress severity as the responses to long term exposure or high stress both produced similar dampened core clock gene rhythmicity. Another explanation that is important to consider is that osmotic stress activates ABA-independent pathways which may produce a distinct effect on expression than that which is produced from ABA alone.

An example of the combined effect of both ABA-dependent and independent re-

sponses brought about by osmotic stress affecting the clock in a manner different than that of ABA alone can be seen with *LUX* expression. *LUX* is a MYB transcription factor that binds to the consensus sequence (GAT(A/T)CG) and is responsible for recruiting the Evening Complex to promoter sequences such as *PRR9*, repressing transcription (Chow et al., 2012). Osmotic stress resulted in strong repression of *LUX* across all time points, an effect which was not observed in response to ABA application. *LUX* has been shown to be regulated via the ABA-independent pathway with CBF1 binding to the single CRT/DRE motif within its promoter (Chow et al., 2014) and over expression of *CBF1* led to increased *LUX* expression levels.

However, despite ABA functioning in combination with ABA-independent responses regarding the effect of osmotic stress on clock gene expression, we found that ABA biosynthesis is necessary for the effect of osmotic stress on circadian leaf movement. It was previously known that ABA decreases the amplitude of leaf movement in *Oxalis regnellii* (purple shamrock) but does not affect period (Skrove et al., 1982). We showed that osmotic stress abolished rhythmic leaf movement in *Arabidopsis* and that this response was dependent on the ability to biosynthesise ABA.

Having identified a strong effect on the expression of *LHY* we were then able to identify the *LHY* promoter G-box as an essential motif for the effect of osmotic stress on the observed circadian damping. ABF4, DPBF2 and ABF3 had perviously been identified as binding to the *LHY promoter* (Kim et al., 2002; Kang et al., 2002). These ABFs have been shown to target G-box containing sequences (Choi et al., 2000) whilst ABF3 was shown to bind to the *LHY* G-box in yeast-1-hybrid experiments (Davies and Carré unpublished).

In conclusion our results are consistent with the hypothesis that ABA plays a role in altering the function of the circadian clock in response to osmotic stress.

Chapter 5

Effect of LHY on ABA Responsive Gene Expression

5.1 Introduction

Chapters 3 and 4 focused on the integration of ABA and osmotic stress signalling into the clock architecture. The data presented in this chapter begins to explore the role of LHY in regulation of ABA biosynthesis and signalling.

5.1.1 Aims

The aims were as follows:

(i) Investigate the effect of LHY on ABA responsive gene expression

(ii) Investigate the effect of LHY on ABA biosynthesis

(iii) Investigate the effect of LHY on ABA related phenotypes

5.2 Results

5.2.1 Interaction of the circadian clock with the ABA signalling pathway

Previous data showed that TOC1 regulates expression of an ABA binding protein (ABAR) (Legnaioli et al., 2009). We exploited available chromatin immunoprecipitation (ChIP) assays with sequencing (ChIP-seq) data to investigate whether other circadian clock genes bound directly to the *ABAR*, genes involved in ABA biosynthesis or the ABA signalling pathway (see table 5.1). PRR7 bound to none of the genes investigated (Liu et al., 2013). TOC1 bound to two targets, a negative regulator of the ABA signalling pathway, protein phosphatase *PP2CA* as well as the aforementioned *ABAR* (Gendron et al., 2012). PRR5 also bound to the *ABAR* as well as two members of the PP2C family (Nakamichi et al., 2010). CCA1 bound to many different genes including *NCED4* involved in ABA biosynthesis but did not bind to the *ABAR* (Nagel et al., 2015). LHY bound to the largest number of genes including the ABAR (Dr. Sally Adams, Dr. Siren Veflingstad and Dr Isabelle Carré). These findings support a strong link between the circadian clock and regulation of the ABA signalling pathway.

		LHY	CCA1	PRR7	PRR5	TOC1
NCED1	AT3G63520					
NCED2	AT4G18350					
NCED3	AT3G14440					
NCED4	AT4G19170		BINDS			
NCED5	AT1G30100					
NCED6	AT3G24220					
NCED9	AT1G78390					
PP2C:HAI1	AT5G59220				BINDS	
PP2C: HAI2	AT1G07430	BINDS	BINDS			
PP2C: HAI3	AT2G29380					
PP2CG1	AT2G33700	BINDS				
PP2C5	AT2G40180					
PP2CA	AT3G11410	BINDS			BINDS	BINDS
SnRK2.2	AT3G50500	BINDS	BINDS			
SnRK2.3	AT5G66880	BINDS				
SnRK2.6	AT4G33950					
ABF1	AT1G49720					
ABF2	AT1G45249					
ABF3	AT4G34000					
ABF4	AT3G19290					
AtHB6	AT2G22430		BINDS			
ABI5	AT2G36270	BINDS	BINDS			
ABI3	AT3G24650	BINDS				
PYR1	AT4G17870					
PYL1	AT5G46790					
PYL2	AT2G26040					
PYL3	AT1G73000					
PYL4	AT2G38310					
PYL5	AT5G05440		BINDS			
PYL6	AT2G40330					
PYL7	AT4G01026	BINDS	BINDS			
PYL8	AT5G53160	BINDS	BINDS			
PYL9	AT1G01360					
PYL10	AT4G27920					
PYL11	AT5G45860					
PYL12	AT5G45870					
PYL13	AT4G18620					
ABAR	AT5G13630	BINDS			BINDS	BINDS

Table 5.1 – Circadian clock genes bind directly to components of the ABA biosynthesis and signaling pathways. Data sourced from ChIP experiments; LHY (Adams and Carré unpublished), CCA1 (Nagel et al., 2015), PRR7 (Liu et al., 2013), PRR5 (Nakamichi et al., 2010) and TOC1 (Gendron et al., 2012).

5.2.2 LHY regulates ABA responsive genes

To begin to investigate whether LHY regulates ABA responsive genes we utilised two unpublished data sets by Dr. Sally Adams, Dr. Siren Veflingstad and Dr Isabelle Carré. The first data set contained results from two gene expression experiments in plants which contained *LHY* under an ethanol responsive promoter (*alc*::*LHY*), allowing for inducible expression of *LHY*. In the first experiment *LHY* expression was induced at ZT17 and differential gene expression assayed via microarray at 2, 4 or 8 hours after induction. The second experiment focused on the response of a selected set of 100 genes when *LHY* was induced across the day. Differential gene expression was assayed 2 hours after ethanol induction via nanostring technology. Genes that were differentially expressed in either or both experiments were identified as candidate regulatory targets for LHY. This resulted in a genome wide list of 6125 genes that were regulated in response to *LHY* expression. The second data set was data from a ChIP-seq experiment which identified direct *LHY* binding targets with a q-value (i.e, corrected p value) $\geq$2. These two data sets were used to reveal a set of 2597 confirmed binding targets who's promoter sequences were directly bound by LHY and also showed differential expression in response to *LHY* induction. All gene lists described in this section are provided on CD as supplementary data.

In order to produce a core list of consistently up-or downregulated genes in response to ABA, a list of ABA regulated genes was compiled by retrieving those which showed 2-fold up-or downregulation in response to ABA from the Transcriptional Regulation By ABA signaling (TRABAS) database (Choudhury and Lahiri, 2008). This list was then filtered against averaged fold change data from 5 separate publicly available experimental data sets exploring the effect of ABA on *Arabidopsis* seedlings (see Table 2.2). Genes which were present in both TRABAS and publicly available lists (Mizoguchi et al., 2010; Pandey et al., 2010; Kim et al., 2011; Umezawa et al., 2013) above a 2-fold threshold were retained.

A hypergeometric analysis (Falcon and Gentleman, 2008) was performed in order to determine over representation of ABA regulated genes. This calculates the probability of k successes in n draws without replacement from a finite population size N. Applied here it describes the probability of finding ABA regulated genes (k) in the list of LHY regulated genes (n) against the whole genome (N). The *Arabidopsis* Information Resource (TAIR10) was used to provide the number of genes within the whole genome (33602). This revealed that ABA regulated genes are extremely over-represented within the set of LHY regulated genes as well as the set of LHY direct binding targets with 88.7 % present in the LHY regulated gene set (see figure 5.1a). ABA regulated genes are also significantly over-represented within the LHY direct binding targets data set (52.1 %) (see figure 5.1b). This suggests a strong link between *LHY* and ABA responsive genes.

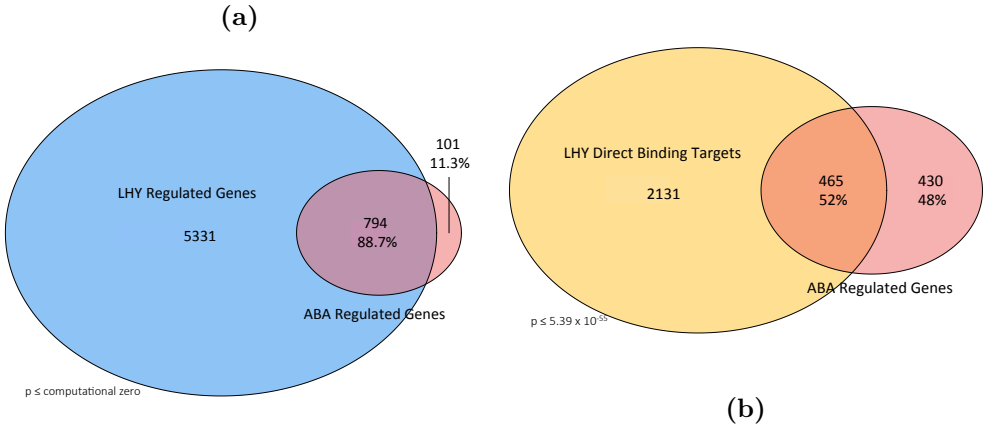

Figure 5.1 – ABA regulated genes are overrepresented within *LHY* regulated genes and direct targets of LHY The "LHY Regulated Genes" set contains those genes which were found to be up or downregulated (over 2-fold) by LHY in microarray experiments using plants containing *LHY* under an ethanol inducible promoter. The "LHY Direct Binding Targets" set contains genes which were identified as direct binding targets of LHY via ChIP-seq experiments. The "ABA Regulated Genes" set contains genes which show a minimum over a 2-fold change in expression in response to ABA. This set was compiled from the TRABAS database and further filtered against 5 individual experiments on *Arabidopsis* seedlings (Mizoguchi et al., 2010; Pandey et al., 2010; Kim et al., 2011; Umezawa et al., 2013). Hypergeometric analysis was performed to determine significant over representation between **a)** LHY regulated genes and ABA regulated genes **b)** LHY direct binding targets and ABA regulated genes

We then refined the "LHY Direct Binding Targets" data set by sorting those which were upregulated and those which were downregulated into separate lists: "LHY Direct Binding Targets upregulated" and "LHY Direct Binding Targets downregulated". The ABA regulated data set was similarly sorted on up-or downregulation in response to ABA. Analysing these data sets by hypergeometric testing showed that the responses to LHY and ABA were not always of the same sign (see Figure 5.2) suggesting that LHY and ABA may both have agonistic and antagonistic effects on the regulation of gene expression.

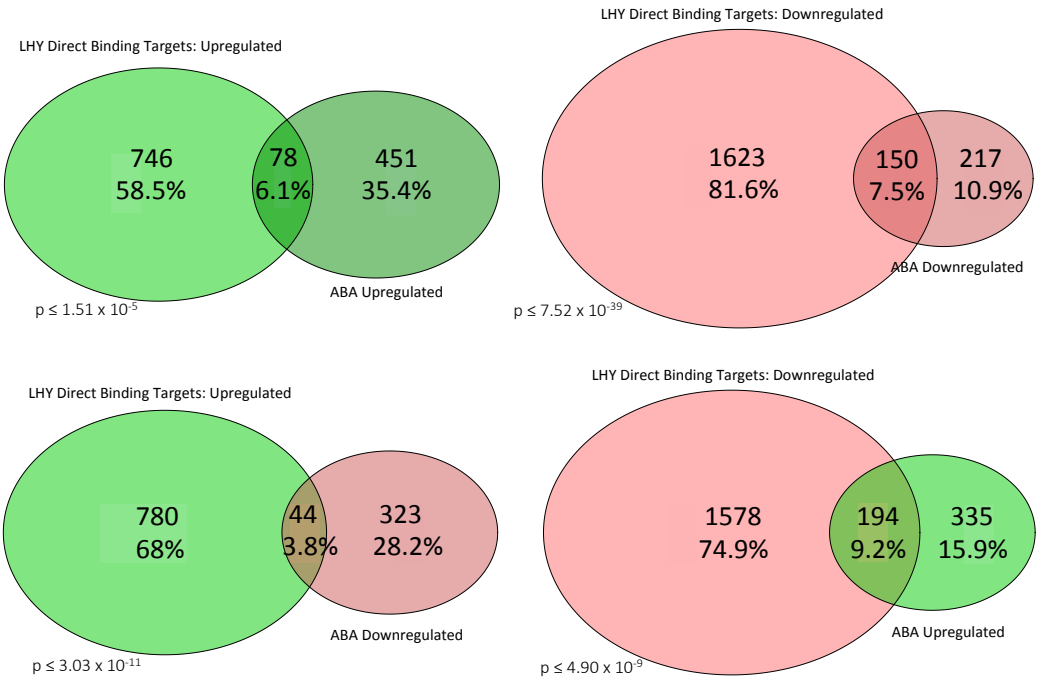

Figure 5.2 – LHY and ABA do not always produce agonistic responses in the genes they regulate Both the "LHY Direct Targets" and "ABA Regulated Genes" sets were sorted based on the direction of their regulation in response to LHY or ABA. Significant over representation was observed in both agonistic and antagonistic combinations via hypergeometric analysis. This suggests a more complex mechanism of interaction between LHY and ABA than a simple positive feedback loop.

5.2.3 Overexpression of *LHY* heightens ABA responsive gene expression

As LHY binds to many components of the ABA signaling pathway we hypothesised that this could potentially affect ABA responsive gene expression by altering the sensitivity of the pathway. We tested the effect of *LHY* overexpression (in *LHY-OX* plants) and loss of function (in the *lhy-11* mutant) on acute responses to ABA application using gene expression as a proxy for ABA responsiveness. In order to identify a suitable set of genes we used the previously described core list of consistently up or downregulated ABA responsive genes and identified those which were stably upregulated whilst also expressed in seedlings (see Table 5.2). It was important that the selected genes were not direct binding targets of LHY so that ABA signalling could be measured without the influence of direct regulation by LHY. Of the four candidate genes identified, the three most responsive were selected for screening (*RD29A*, *LEA7* and *LTI30*). Experiments were carried out as described in Chapter 3; seedlings were grown under a 12L 12D light regimen for 7 days then transferred to constant light. ABA or vehicle (methanol) was sprayed onto the seedlings at either ZT24, 27, 30, 33, 36, 39, 42 or 45. Samples were harvested 3 hours after treatment and flash frozen in liquid nitrogen for RNA extraction, cDNA synthesis and analysis by qPCR. Gene expression levels were calculated relative to *ACTIN2*.

ABA response	ATG Code	Name	GO Biological Process
Upregulated	AT5G52310	RD29A	hyperosmotic salinity response, leaf senescence, regulation of root development, response to abscisic acid, response to cold, response to desiccation, response to mannitol, response to osmotic stress, response to reactive oxygen species, response to salt, response to salt stress, response to symbiotic bacterium, response to water deprivation, response to wounding
Upregulated	AT1G52690	LEA7	embryo development ending in seed dormancy
Upregulated	AT3G50970	LTI30	cold acclimation, defense response to fungus, response to abscisic acid, response to cold, response to water, response to water deprivation
Upregulated	AT3G02480	ABR	response to abiotic or biotic stimulus, response to stress

Table 5.2 – ABA and osmotic stress responsive genes used as proxies for assaying the status of ABA signaling and ABA responsiveness. ABA responsiveness was determined from 5 publicly available data sets (Mizoguchi et al., 2010; Pandey et al., 2010; Kim et al., 2011; Umezawa et al., 2013) as described in section 5.2.1. Gene ontology (GO) descriptors were obtained from The Arabidopsis Information Resource (TAIR). None of the above genes are direct targets of LHY allowing measurement of ABA signaling without influence from direct regulation by LHY.

LHY-OX had greater induction of *RD29A* compared to the wild type at all times of the day (see Figures 5.3a and b). The most significant ($p \leqslant 0.001$) of these were ZT27, 42, 45 and 48, time points corresponding to late night and subjective dawn. *lhy-11* had a reduced level of induction at all time points tested except ZT30. Basal expression of *RD29A* was also lower in *lhy-11*, while the *LHY-OX* line had significantly higher levels compared to the wild type. This may reflect responses to endogenous ABA.

Similar observations were made for *LATE EMBRYOGENESIS ABUNDANT 7* (*LEA7*) and *LOW TEMPERATURE INDUCED 30* (*LTI30*) levels (see Figures 5.3c, d, e

and f). The *lhy-11* line showed significantly reduced induction at all time points tested. *LHY-OX* had significantly higher induction for the majority of time points tested. In the control condition both *lhy-11* and *LHY-OX* had significantly lower basal expression levels than the wild type for the majority of time points tested.

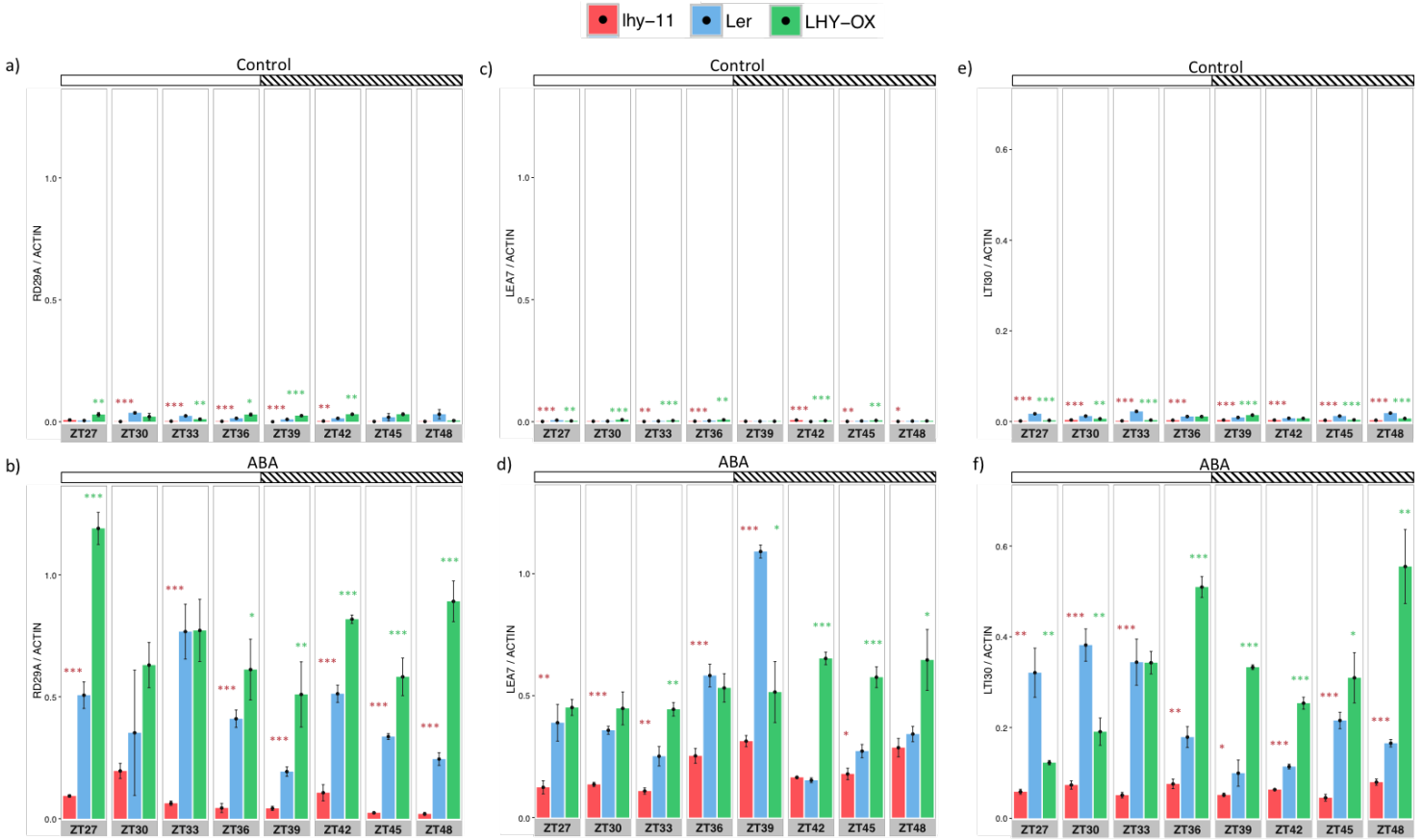

Figure 5.3 – Increased LHY results in increased ABA responsive gene expression, decreased LHY levels result in decreased expression *lhy-11* (Ler), *LHY-OX* (Ler) and wild type (Ler) seedlings were grown under a 12L 12D light regimen for 7 days then transferred to constant light. 25 µM ABA or vehicle (methanol) was sprayed onto plants at one of the following time points (ZT24, 27, 30, 33, 36, 39, 42 or 45), harvested 3 hours after and flash frozen in liquid nitrogen for RNA extraction, cDNA synthesis and analysis by qPCR. **a) and b)** *RD29A*, **c) and d)** *LEA7* and **e) and f)** *LTI30* gene expression levels were calculated relative to *ACTIN2*. Data represents the mean of technical triplicates for a single biological replicate with error bars showing standard deviation. Results were consistent across 3 separate biological replicates. Asterisks indicate p-values from T-tests comparing mutant genotypes to wild type. Green asterisks show the significance between *LHY-OX* and Ler whilst red asterisk represent the significance between *lhy-11* and the wild type (* $p \leqslant 0.05$; ** $p \leqslant 0.01$; *** $p \leqslant 0.001$)

These results may be explained by either of two hypotheses:

1. LHY promotes ABA biosynthesis

2. LHY promotes stress responsive signaling downstream of ABA

Hypothesis 1 was tested in the following section.

5.2.4 Overexpression of *LHY* reduces ABA hormone biosynthesis

Although LHY does not bind to any of the key ABA biosynthesis *9-CIS EPOXY-CAROTENOID DIOXYGENASE (NCED)* genes directly, we tested the effect of *LHY* mis-expression on *9-CIS EPOXYCAROTENOID DIOXYGENASE 3 (NCED3)*. The NCED family is responsible for the oxidative cleavage of violaxanthin to xanthoxin at the start of the dedicated ABA biosynthesis pathway and mis-regulation could therefore result in altered levels of ABA biosynthesis.

Seedlings were grown for 7 days under 12L 12D cycles before transferring to constant light. 25 μM ABA or control was then sprayed onto seedlings at ZT0 and tissue harvested 3 hours later. *LHY-OX* had significantly reduced levels of *NCED3* expression in both control and ABA treated conditions (see Figure 5.4).

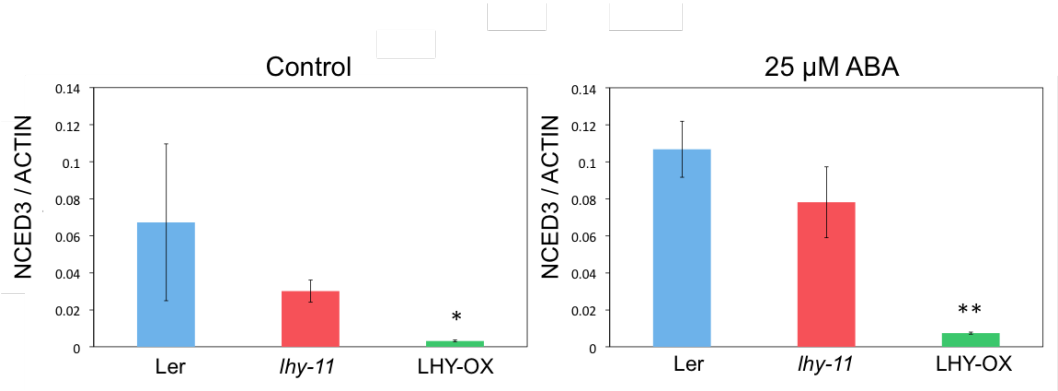

Figure 5.4 – Overexpression of *LHY* results in reduced NCED3 expression in the presence and absence of ABA *Arabidopsis* seedlings (Ler) were grown on MS0 for 7 days at 22°C and entrained to a 12L 12D lighting regimen before being transferred to constant light. 25 µM ABA or control was then sprayed onto seedlings at ZT0 and tissue harvested 3 hours later and flash frozen. *NCED3* transcript levels were analysed by qPCR and quantified relative to *ACTIN2* expression. Data represents the mean of technical triplicates with error bars showing standard deviation. Asterisks indicate p-values from T-tests comparing control and treated conditions (* $p \leqslant 0.05$; ** $p \leqslant 0.01$; *** $p \leqslant 0.001$).

We then tested whether this translated to an effect on ABA hormone levels. *lhy-11*, *LHY-OX* and Landsberg seedlings were grown in a randomised configuration on soil and entrained to 16L 8D light cycles. Over the first 14 days of growth plants in both the watered and drought conditions received water on every third day, after this period watering was withheld entirely from the drought stress condition. After a further 10 days of growth, rosette samples were harvested at 3 hour intervals across a 24 hour period and flash frozen. Tissue was then processed and analysed via HPLC-electrospray ionisation MS/MS to quantify ABA hormone levels.

No significant difference was observed between genotypes in the watered condition, all showing low ABA levels between 0-10 ng per gram of tissue. In the drought condition, the wild type contained low ABA levels over the morning period which began to rise through the day before peaking at 562 ng per gram just after nightfall (see Figure 5.5). The timing and quantity of this peak conforms exactly with perviously reported data on which this detection method was based and allows us to

have confidence in the dataset (Forcat et al., 2008). The *lhy-11* line performs similarly to the wild type although the peak of ABA level only reaches 399 ng per gram and occurs 3 hours earlier. Consistent with the advanced phase phenotype of the mutant the level then rapidly drops with ZT18 being the only significantly different ($p \leqslant 0.05$) time point from the wild type. The *LHY-OX* line had consistently low levels of ABA and was significantly different from the wild type at all mid day and evening time points.

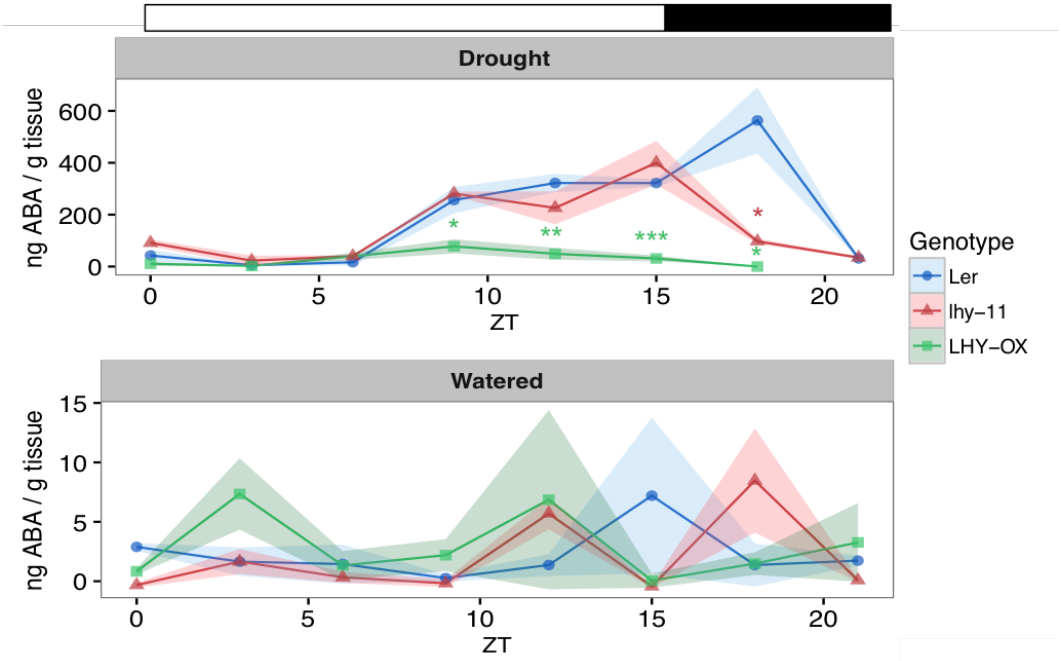

Figure 5.5 – Overexpression of *LHY* results in lower ABA hormone levels in drought conditions. *lhy-11*, *LHY-OX* and Landsberg seedlings were grown in a randomised configuration on soil and entrained to 16L 8D light cycles. Over the first 14 days of growth plants in both the watered and drought conditions received water on every third day, after this period watering was withheld entirely from the drought stress condition. After a further 10 days of growth, rosette samples were harvested at 3 hour intervals across a 24 hour period and flash frozen. Tissue was then processed and analysed via HPLC-electrospray ionisation MS/MS to quantify ABA hormone levels. White and black bars represent subjective day and night, respectively. Data represents the mean from 3 technical triplicates for a pooled sample of 2 biological replicates, error bars showing standard deviation. Asterisks indicate p-values from T-tests comparing mutant genotypes to the wild type. Green asterisks show the significance between *LHY-OX* and Ler whilst red asterisks represent the significance between *lhy-11* and the wild type (* $p \leqslant 0.05$; ** $p \leqslant 0.01$; *** $p \leqslant 0.001$)

5.2.5 Characterisation of *pLHY* transgenic lines

Three complemented *lhy-11 LHY:LHY* lines, in which the *LHY* coding region had been re-introduced to the *lhy-11* background under the control of its own promoter (*pLHY1, pLHY2, pLHY3*) were generated by Dr. Jae-Yean Kim. In order to determine the levels of LHY, protein levels were quantified via western blotting (see Figure 5.6a.i). Seedlings were grown for 14 days under 12L 12D cycles and tissue was harvested at ZT1 to coincide with the peak of LHY protein level. Ponceau staining was performed to ensure equal loading of samples in the SDS gel (see Figure 5.6a.ii). Band density was calculated relative to the wild type (Ler) (see Figure 5.6a.iii). This revealed that expression of the construct was successful in all three lines with the LHY level in *pLHY1* being most similar to the wild type, although slightly higher. *pLHY2* and *pLHY3* had higher levels than both *pLHY1* and the wild type with *pLHY3* levels resembling that of the *LHY-OX* line.

We also performed a luciferase time course to observe the rhythmic circadian expression of *LHY*::*luc* which was inserted on the same plasmid as the *pLHY*::*LHY* fusion. The *lhy-11* line contains an *LHY*::*luc* construct and is included as a control. Seedlings were grown for 7 days under 12L 12D cycles and imaged using a photon counting camera. All lines tested show rhythmic peaks occurring at dawn as expected (see Figure 5.6b). *LHY* expression in the *lhy-11* line begins prior to that of the wild type (Ler) which is expected as shortened period leads to earlier phase onset in entrained conditions. In contrast, *pLHY2* and *pLHY3* (both with higher *LHY* expression levels than the wild type) show delayed phase of *LHY* expression onset than the wild type with a delayed return to baseline levels. Increased *LHY* corresponded to reduced amplitude of rhythmic expression indicating increased LHY function.

These results reveal a gradient of *LHY* expression that increases incrementally from:

the knockout *lhy-11*, wild type (Ler), *pLHY1*, *pLHY2*, *pLHY3* and *LHY-OX*.

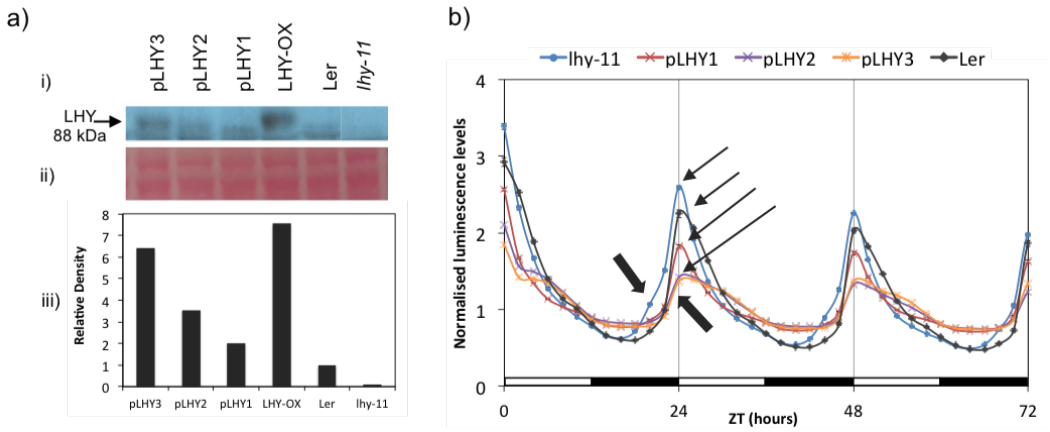

Figure 5.6 – All *lhy-11 LHY::LHY* complemented lines express LHY protein at levels equal or above the wild type at ZT1. a) Ler (WT), *LHY-OX*, *pLHY1, 2* and *3* seedlings were grown for 14 days under a12L 12D light regimen. Samples were harvested at ZT1 so as to coincide with the peak of LHY protein expression. LHY abundance was quantified in each line via western blot (i) and a Ponceau stain preformed to verify equal loading of sample (ii). Band density was then calculated relative to the wild type (iii). **b)** Seedlings were grown for 7 days under a 12L 12D lighting regimen and then imaged with a photon counting camera. The *lhy-11* line contains an *LHY*::*luc* construct and is included as a control. Open and black bars represent day and night, respectively. Thick arrows highlight the differences in onset of *LHY* expression at dawn whilst thin arrows highlight the differences in amplitude. Data are means from at least 6 independent replicates, in at least two independent experiments. Temporal patterns of luminescence were normalised to the mean expression level in constant light. Error bars indicate the Standard Error of the Mean (SEM).

5.2.6 LHY levels affect seed germination in response to exogenous ABA application

ABA acts antagonistically to gibberellic acid resulting in delayed germination in wild type seed (Debeaujon and Koornneef, 2000; Koorneef et al., 1985). Because of this, ABA-deficient mutants have been identified in a variety of species through germination assays (Schwartz et al., 1997*b*; Koornneef et al., 1982). We decided to use a similar experimental design to investigate whether LHY affected responses to ABA.

When grown on MS0 there were no significant differences in germination percent-

age with Ler, *lhy-11* and *LHY-OX* lines all reaching maximal germination 4 days after transfer to 22 °C (see Figure 5.7a). *pLHY2* and *pLHY3* had reduced viability, having only reached 83.3 % and 63.8 % after 6 days, respectively. In the presence of 0.5 μM ABA the wild type (Ler) line germinated at a slower rate than that of the control condition reaching 33.3 % 6 days after stratification. In comparison the *LHY-OX* line performed significantly better ($p \leqslant 0.05$) and reached 94.4 % whilst no seeds had germinated in the *lhy-11* line (see Figure 5.7b). Total germination percentages comparable to that of the control condition were reached for both *LHY-OX* and the wild type although this required 7 and 9 days respectively. After 10 days the *lhy-11* line had only reached 3.6 %. This trend was also observed in response to 2 μM ABA where the *LHY-OX* line achieved 72.2 % total germination after 6 days and reached total germination after 8 days (see figure 5.7c). The wild type showed 9.25 % after 6 days days and only reached 63.8 % after the 10 day period. *lhy-11* achieved 4.6 % after 6 days and 11 % after 10 days which is significantly lower ($p \leqslant 0.01$) than the wild type. *pLHY1* performed similarly to the *lhy-11* line with only 2.7 % after 6 days and reaching a maximum of 16.6 % after 10 days. *pLHY2* only managed to reach a maximum of 75 % germination after 10 days. *pLHY2* line performed better than *lhy-11* with 5.5 % after 6 days and 33.3 % after 10 days. *pLHY3* behaved similarly to the wild type with 30.5 % after 6 days and 55.5 % after 10.

These results suggest that elevated expression of *LHY* acts to reduce sensitivity to ABA in the context of germination. Comparing performance across ABA treatment concentrations 6 days after stratification highlights this, with *LHY-OX* performing significantly better than the wild type and the *lhy-11* line performing significantly worse (see Figure 5.7d).

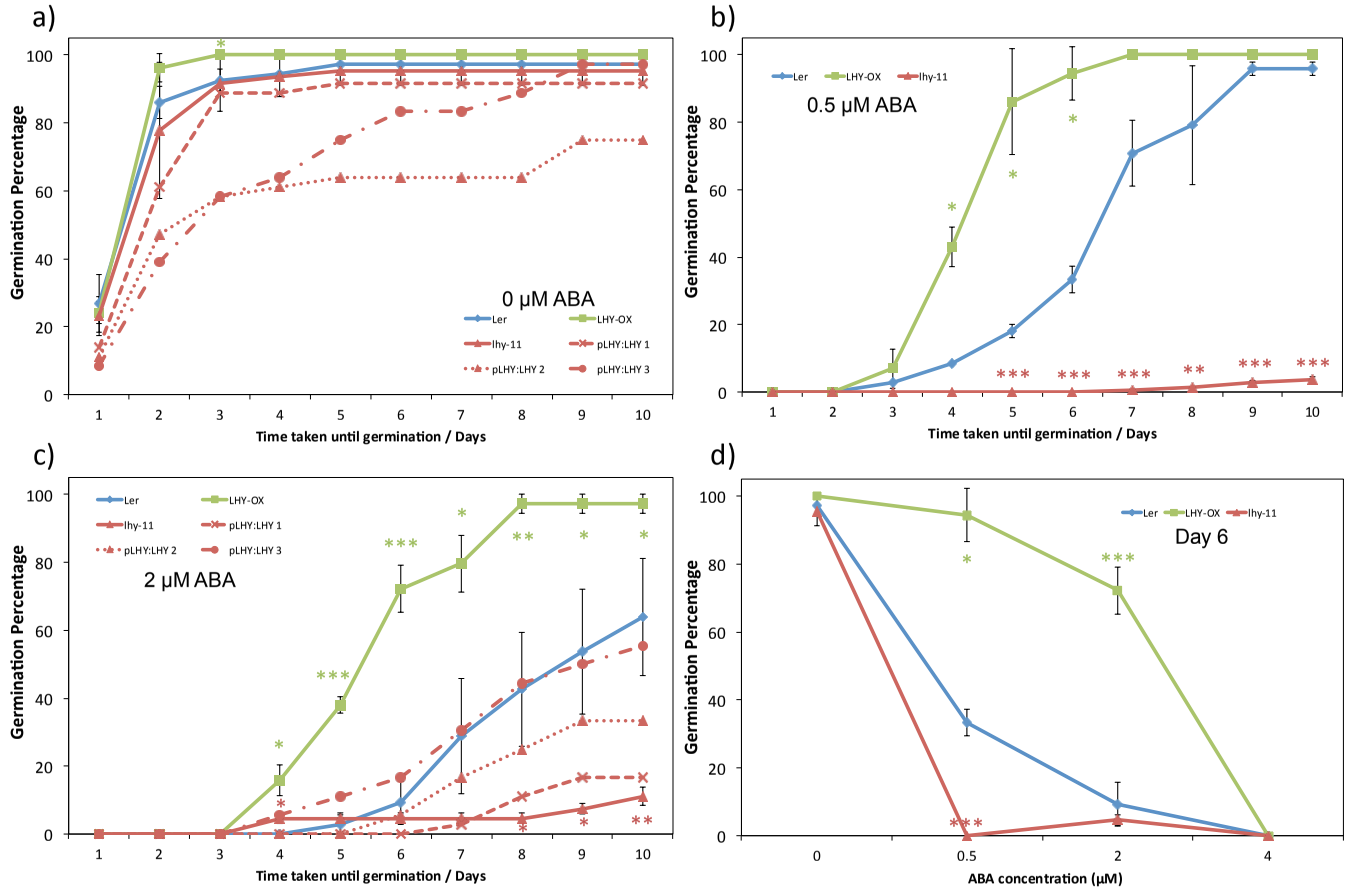

Figure 5.7 – LHY reduces sensitivity to ABA in the context of germination. *Arabidopsis* plants for seed production were grown together and harvested simultaneously. Seeds were then placed individually onto either MS0 media **a)** or media containing 0.5 µM ABA **b)**, 2 µM ABA **c)**. Plates were stratified before being transferred to consent light. Germination was scored daily using a microscope to detect radical emergence. Data represents the mean percentage of germination across 6 independent progenies from individual plants for Ler, *lhy-11* and *LHY-OX* lines and one for each of the *pLHY::LHY* complemented lines. **d)** Shows germination percentages for Ler, *lhy-11* and *LHY-OX* lines across all ABA concentrations tested 6 days after stratification. T-tests were performed to evaluate statistical significance. Green asterisks show the significance between *LHY-OX* and Ler whilst red asterisks represent the significance between *lhy-11* and the wild type (* $p \leqslant 0.05$; ** $p \leqslant 0.01$; *** $p \leqslant 0.001$).

5.2.7 Increased *LHY* expression mitigates the negative effect of ABA on seedling growth

Using an *in vitro* phenotyping system, `rosettR` (Redestig, 2016; Tomé et al., 2016) developed at the Bayer CropScience division in Ghent, Belgium, we investigated whether *LHY* levels would affect the growth of seedlings in the presence of ABA. Landsberg, *LHY-OX*, *lhy-11*, and the *pLHY1, 2, 3* lines were sown onto plates in a grid formation on top of nylon membranes. The `rosettR` package for R was used to determine the layout of the plates and the randomisation of experimental blocks. After 10 days of growth under 12L: 12D cycles, seedlings were transferred to fresh media containing either 2 µM or 10 µM ABA. Control plates were transferred to fresh MS0 media. Growth continued under 12L:12D cycles for a further 8 days. Photographs were then taken daily in order to quantify seedling growth over time.

Strong overexpression of *LHY* also results in a developmental phenotype with elongated petioles and hypocotyl, producing a seedling with smaller surface area during early stages of growth. This is evident in the smaller total average seedling area the *LHY-OX* line reached during the growth period.

No significant differences were observed in any of the genotypes tested in response to 2 µM ABA. However, the wild type showed significant reduction in average seedling area in the presence of 10 µM ABA whilst the *lhy-11* and *LHY-OX* lines showed a reduced sensitivity to these treatments (see Figure 5.8). *pLHY1*, *pLHY2* and *pLHY3* all performed better than the wild type in the presence of 10 µM ABA. *pLHY2* and *pLHY3* showed no significant difference between the control and experimental treatments at any time point. These results would suggest that an increase in *LHY* expression results in reduced sensitivity to abiotic stress on growth in seedlings.

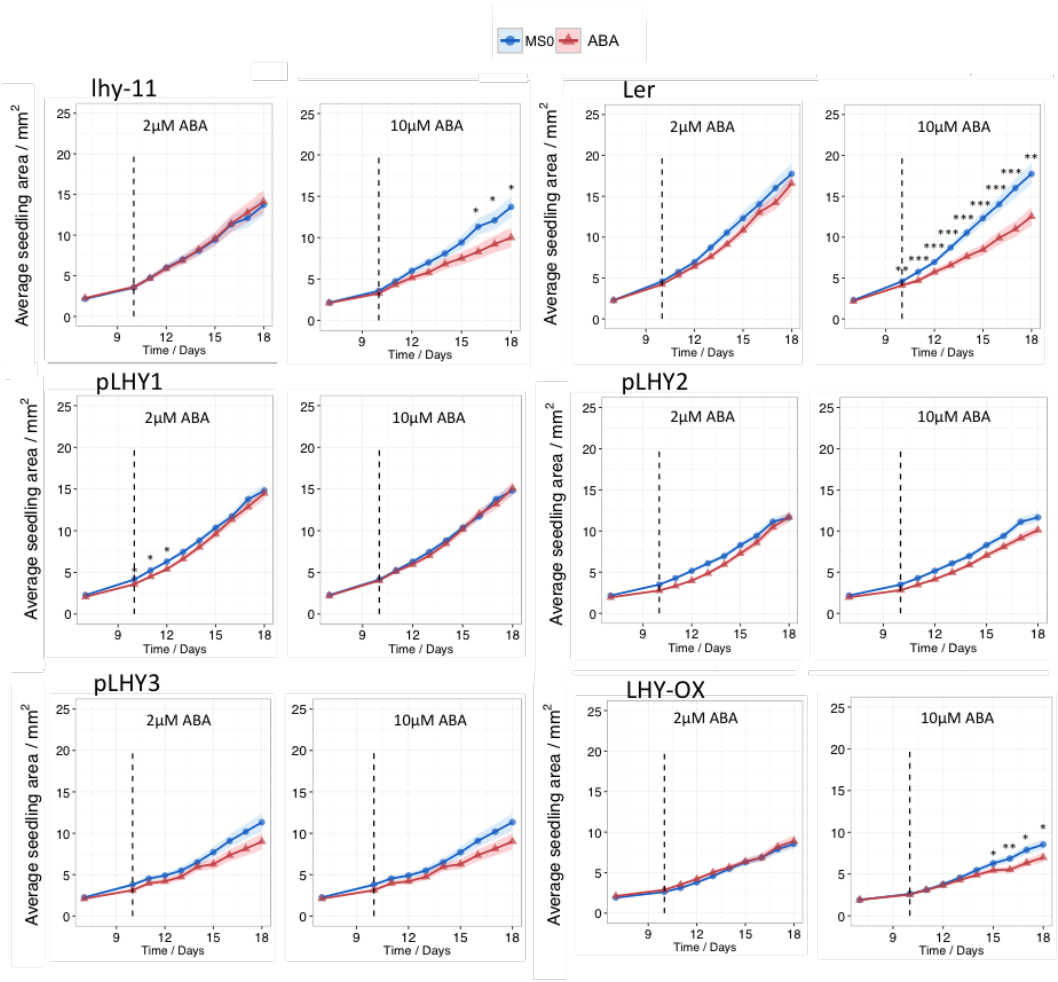

Figure 5.8 – Increased *LHY* expression results in reduced effect of ABA on seedling growth Seedlings were grown for 18 days under 12L 12D cycles. After 10 days of growth on MS0 seedlings were transferred to either fresh MS0 or MS0 containing ABA (2 μM or 10 μM). Aerial photographs were taken daily and used to calculate average seedling size using the `rosettR` package in R. Data represents the means from 192 biological replicates across 2 independent experiments. Standard deviation is indicated by the colour ribbon for each treatment. Asterisks indicate p-values from T-tests comparing experimental treatments to the control condition at each time point (* $p \leqslant 0.05$; ** $p \leqslant 0.01$; *** $p \leqslant 0.001$).

5.2.8 Discussion

Through analysis of publicly available data sets we have revealed that TOC1, PRR5, CCA1 and LHY bind to the promoters of components of the ABA signaling pathway. This suggests that the clock may influence ABA perception which may help

account for the time of day dependent responses to abiotic stress (Kiełbowicz-Matuk et al., 2014). However, the combined effect of clock genes binding to ABA signaling components remains unclear. PRR5 and TOC1 are revealed to bind to both the ABAR and one member of the PP2C family. TOC1 has been shown to be a DNA binding protein which represses gene expression (Gendron et al., 2012) and has been shown to repress *ABAR* expression (Legnaioli et al., 2009). Reduction of PP2C levels would be expected to sensitise responses to ABA by reducing the phosphatase activity required to inhibit the SnRK kinases. Contrastingly, reduction in ABAR levels would appear to desensitise responses to ABA, if it does indeed function as part of an ABA signalling pathway as opposed to just binding ABA. Interpretation becomes even more complicated for LHY and CCA1 as they bind to many components of the ABA signalling pathway including both positive and negative regulators.

Germination assays revealed that increased *LHY* expression decreased sensitivity to ABA allowing faster germination in the presence of ABA, whereas a reduction of *LHY* expression levels resulted in heightened sensitivity and prolonged dormancy in the presence of ABA. It is well established that ABA plays a prominent role in seed development and the regulation of germination (Kermode, 2005). During development ABA biosynthesis encourages seed maturation through the continued production of storage proteins and lipids (Karssen et al., 1983). Acting in balance with gibberellin (GA) it also prevents premature germination, prolonging dormancy (Karssen and Lacka, 1986). Prolonged dormancy can be observed in mutants which over-accumulate ABA (Thompson, Jackson, Symonds, Mulholland, Dadswell, Blake, Burbidge and Taylor, 2000; Okamoto et al., 2006). Immature seeds are able to receive ABA from maternal tissues in addition to synthesising their own. With this in mind, an initial explanation for the reduced dormancy observed in the *LHY-OX* line could be due to the reduced activity of *NCED3* and the reduced ABA hormone levels observed in response to drought. The quantity of ABA detected and the tim-

ing of the peak level of expression in the wild type line corresponded very nicely with those previously reported (Forcat et al., 2008) making this data particularly convincing. However, this is not sufficient to explain the differences observed in the presence of exogenous ABA as each line is exposed to the same external ABA concentration. Furthermore, ABA hormone levels were not found to be elevated above the wild type in the *lhy-11* line and would not explain the increased level of dormancy observed. While these results are consistent with the hypothesis that LHY acts to antagonise the effect of ABA in seed, other explanations are possible. Recent work has revealed that late flowering mutants such as *LHY-OX* have altered seed coat tannin content. Increased tannin content was correlated with decreased permeability and thus reduced uptake of externally applied tetrazolium dye (Chen et al., 2014). Mis-expression of *LHY* may alter the formation of the seed coat with overexpression resulting reduced permeability, and knockout increasing permeability in comparison with the wild type. Another contributing factor could be the effect of LHY on ABI5. ABI5 has been shown to bind to ABI3 *in vivo* and that this is important for ABI3 function (Lopez-Molina et al., 2001). As LHY is known to repress gene expression and LHY was shown to bind to the *ABI5* promoter in ChIP-seq experiments, this could reduce ABI3 action and lead to reduced dormancy. It is known that *abi3* mutants are non-dormant. Finally, it is possible that LHY regulates gibberellin synthesis or signalling leading to increased gibberellin hormone levels or pathway sensitivity with increased levels of *LHY* expression.

By using downstream ABA and osmotic stress responsive genes as a proxy we showed that increased LHY levels resulted in enhanced responses to ABA whilst lower LHY levels reduced them. This response was not time of day dependent for *RD29A*, but both *LEA7* and *LTI30* showed greater response in *LHY-OX* during the subjective night phase. Whilst we have shown that biosynthesis of ABA peaks during the night period in wild type plants under long term stress conditions, this

would not be a factor in the time dependent response as ABA was applied exogenously at the same concentration to all samples. Instead, it appears that the gated sensitivity of *LEA7* and *LTI30* in the wild type is disrupted in *LHY-OX* producing a more uniform response across time.

Seedling growth assays revealed that moderate increases in LHY levels (*pLHY1*, *pLHY2* and *pLHY3*) provided the optimum level of plant performance in the presence of ABA. The reduced performance of *LHY-OX* relative to that of the complemented lines may be due to the smaller seedling size of *LHY-OX* plants. Smaller seedling size may amplify the stress of ABA application during early growth stages. Interestingly, knock-out of *LHY* also seemed to confer an advantage to plant performance in regards to seedling growth, performing better than the wild type but worse than the complemented lines. This was not expected given that ABA responsive gene induction was shown to be impaired in *lhy-11*.

As increased LHY levels were also shown to mitigate the inhibitory effect of ABA on both germination and seedling growth and having shown that gene expression responses to LHY and ABA were not always of the same sign we propose that LHY acts agonistically to promote genes responsible for increased tolerance to abiotic stress whilst acting antagonistically to inhibit ABA responsive genes responsible for reduced growth and seed germination (see Figure 5.9). We have also shown that overexpression of *LHY* leads to reduced *NCED3* expression and lower ABA biosynthesis in response to stress. Furthermore, we have shown that ABA responsive genes are over represented amongst LHY binding targets suggesting that LHY regulates the response to ABA on a scale much wider than just that of the ABA signalling pathway.

The results in this chapter provide strong evidence that LHY influences plants'

sensitivity to ABA, which may impact on plant performance under drought and osmotic stress. This will be tested in Chapter 6.

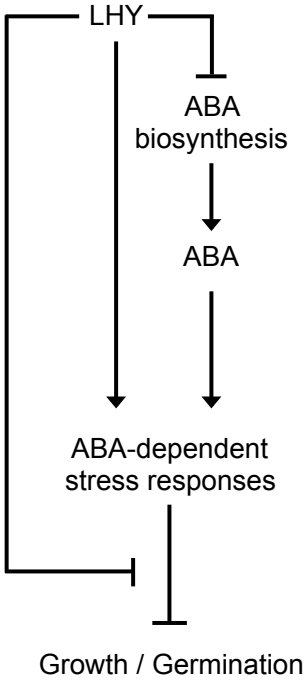

Figure 5.9 – Proposed model for agonistic and antagonistic action of LHY on ABA responses. LHY reduces ABA biosynthesis but enhances ABA-dependent stress responsive genes. LHY acts antagonistically with a sub set of these responsive genes that are responsible for reduced growth and germination in the presence of ABA. In this manner LHY could produce enhanced responses to ABA whilst also mitigating the inhibitory effect of ABA on growth and seed germination.

Chapter 6

Effect of Altered Circadian Clock Function on Phenotypic Stress Responses

6.1 Introduction

Previous research reported the effect of *TOC1* mis-expression on control of stomatal aperture (Legnaioli et al., 2009). Overexpression of *TOC1* resulted in stomatal insensitivity, remaining open in presence of exogenous ABA application whereas reduced levels of *TOC1* expression resulted in heightened stomatal sensitivity, with increased closure. Furthermore, these lines were shown to have altered survival rates to dehydration stress with only 2 % of *TOC1-OX* plants recovering after re-watering compared to 52 % of the TOC1-RNAi. The wild type had an intermediate phenotype with 15 % survival. However, in Chapter 5 it was shown that mis-expression of *LHY* also resulted in altered ABA responsive gene expression. Overexpression of *LHY* produced heightened responses although these did not arise as a result of increased ABA biosynthesis. In order to test whether *LHY* expression levels also impact on plant performance under drought and osmotic stress, the work presented in this

chapter describes phenotypic analysis of plants with altered *LHY* expression.

6.1.1 Aims

The experimental aim was as follows:
Investigate whether altered circadian clock function affects osmotic or drought stress phenotypes

6.1.2 Overexpression of *LHY* heightens ABA responsive gene expression under osmotic stress

We tested whether LHY affected ABA responsive gene expression under osmotic stress conditions. Seedlings were grown on MS0 or MS0 containing 100 mM sorbitol for 7 days entrained to a 12L 12D light regimen before being transferred to constant light. Tissue was then harvested at subjective dawn ZT0 and flash frozen for RNA extraction, cDNA synthesis and analysis via qPCR focusing on ABA responsive gene candidates previously described in 5.2.

In comparison to the wild type, *LHY-OX* showed significantly increased expression in response to osmotic stress for all ABA responsive genes tested (*RD29A*, *LEA7*, *ABR* and *LTI30*) (see Figure 6.1). However, basal expression levels in the control condition were not higher. *lhy-11* was only significantly different from the wild type in *RD29A* expression, which was reduced. These results suggest that overexpression of *LHY* results in a stronger response to osmotic stress and increased ABA responsive gene expression.

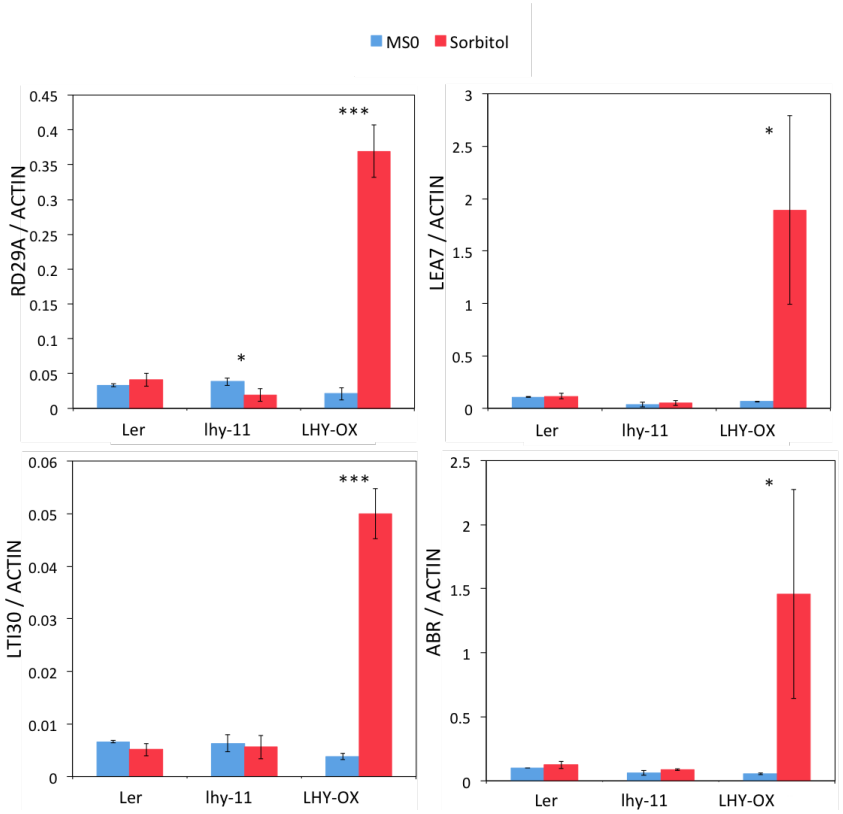

Figure 6.1 – Overexpression of *LHY* results in greater induction of ABA responsive genes in response to osmotic stress. *lhy-11* (Ler), *LHY-OX* (Ler) and wild type (Ler) seedlings were grown on MS0 or MS0 containing 100 mM sorbitol, under a 12L 12D light regimen for 7 days then transferred to constant light. Tissue was the harvested at subjective dawn ZT0 and flash frozen for RNA extraction, cDNA synthesis and analysis via qPCR. Gene expression levels were calculated relative to *ACTIN2*. Data represents the mean of technical triplicates for a single biological replicate with error bars showing standard deviation. Results were consistent across 3 separate biological replicates. Asterisks indicate p-values from T-tests comparing control and osmotic stress conditions for each genotype (* $p \leqslant 0.05$; ** $p \leqslant 0.01$; *** $p \leqslant 0.001$)

6.1.3 Increased *LHY* expression mitigates the negative effect of osmotic stress on seed germination

Having shown that increased *LHY* expression results in reduced sensitivity to exogenous ABA application, we decided to use a similar experimental design to investigate whether *LHY* expression affected germination in the presence of osmotic stress.

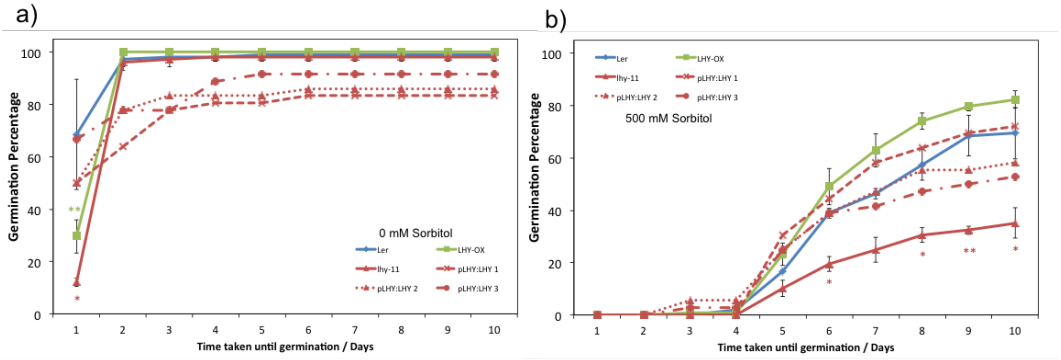

Figure 6.2 – Loss of *LHY* function results in reduced germination under osmotic stress. Seeds were then placed individually onto either MS0 media **a)** or media containing 500 mM sorbitol **b)**. Plates were stratified before being transferred to constant light. Germination was scored daily using a microscope to detect radical emergence. Data represents the mean percentage of germination across 6 independent progenies from individual plants for Ler, *lhy-11* and *LHY-OX* lines and one for each of the *pLHY::LHY* complemented lines. Green asterisks show the significance between *LHY-OX* and Ler whilst red asterisks represent the significance between *lhy-11* and the wild type (* $p \leqslant 0.05$; ** $p \leqslant 0.01$; *** $p \leqslant 0.001$).

In the control condition, the wild type, *lhy-11* and *LHY-OX* lines reached maximum germination percentages 2 days after stratification. The complemented *pLHY* lines showed slower rates of germination and achieved total germination percentages approximately 20 % less than that of the wild type (see Figure 6.2a). Under osmotic stress the wild type showed slower germination reaching a final germination percentage of 69.4 % after 10 days (see figure 6.2b). In comparison, the *lhy-11* line performed significantly worse reaching a maximum germination percentage of 35.2 %. Although the *LHY-OX* line appeared to perform better than the wild type with a greater total percentages each day throughout the time course, these differences were not statistically significant. The *pLHY1, 2* and *3* lines were not significantly different from the wild type suggesting that the *pLHY*::*LHY* transgene had effectively restored normal sensitivity to osmotic stress. These results suggest that loss of *LHY* results in heightened sensitivity to osmotic stress.

6.1.4 Increased *LHY* expression mitigates the negative effect of osmotic stress on seedling growth

Using an *in vitro* phenotyping system, `rosettR` described in Chapter 5, we investigated whether *LHY* levels would affect the growth of seedlings in the presence of sorbitol. Landsberg, *LHY-OX*, *lhy-11*, and the *pLHY1, 2, 3* lines were sown onto plates in a grid formation on top of nylon membranes. The `rosettR` package for R was used to determine the layout of the plates and the randomisation of experimental blocks. After 10 days of growth under 12L: 12D cycles, seedlings were transferred to fresh media containing either 150 mM or 300 mM sorbitol. Control plates were transferred to fresh MS0 media. Growth continued under 12L:12D cycles for a further 8 days. Photographs were then taken daily in order to quantify seedling growth over time.

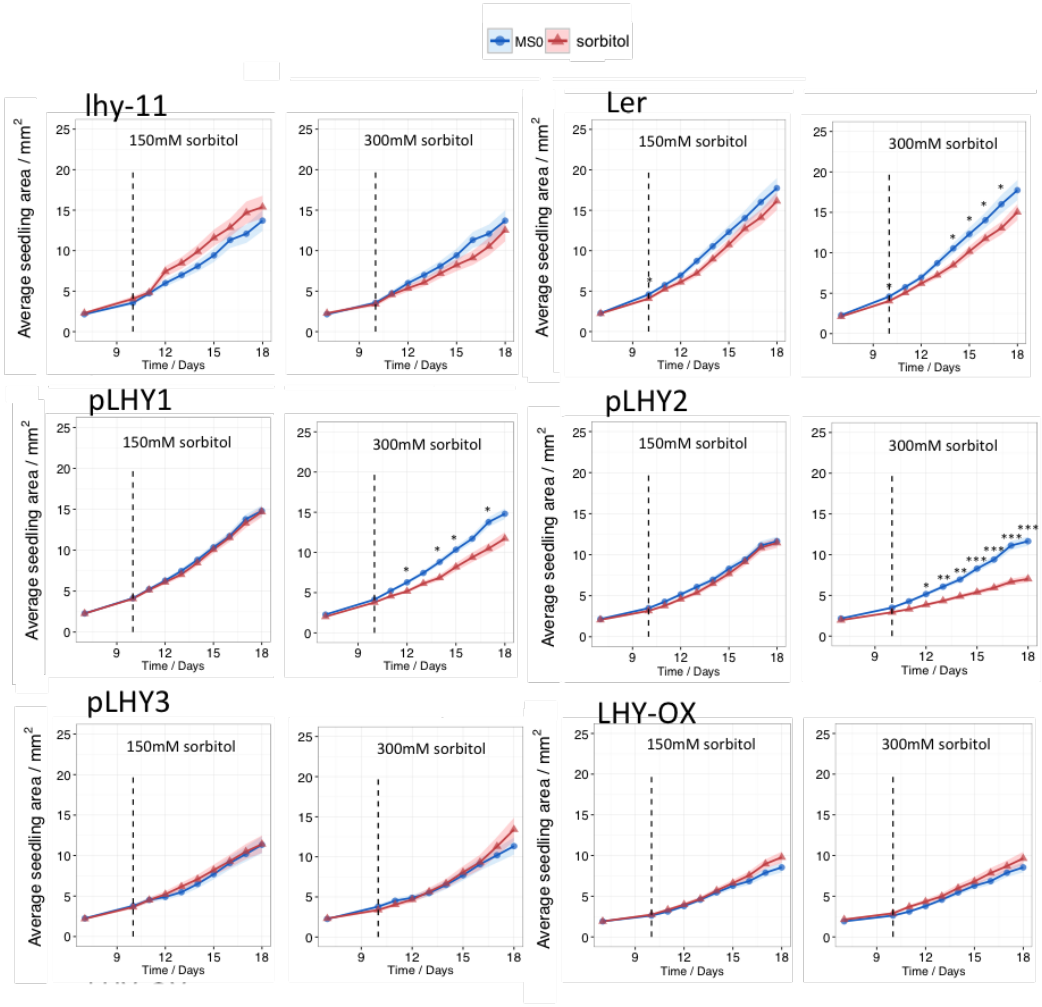

Figure 6.3 – Increased *LHY* expression results in reduced effect of sorbitol on seedling growth Seedlings were grown for 18 days under 12L 12D cycles. After 10 days of growth on MS0 seedlings were transferred to either fresh MS0 or MS0 containing sorbitol (150mM or 300mM). Aerial photographs were taken daily and used to calculate average seedling size using the `rosettR` package in R. Data represents the means from 192 biological replicates across 2 independent experiments. Standard deviation is indicated by the colour ribbon for each treatment. Asterisks indicate p-values from T-tests comparing experimental treatment to the control condition at each time point (* $p \leqslant 0.05$; ** $p \leqslant 0.01$; *** $p \leqslant 0.001$).

No significant differences in average seedling area were observed in any genotype tested in response to 150 mM sorbitol (see Figure 6.3). *lhy-11*, *pLHY3* and *LHY-OX* also showed no significant difference in response to 300 mM sorbitol. However, the wild type (Ler) showed significant ($p \leqslant 0.05$) reduction in average seedling area

between days 14 and 18. *pLHY1* was also affected with significant ($p \leqslant 0.05$) differences detected after 12 days of growth. *pLHY2* was the most affected line with effects also detected after 12 days of growth although continuing with greater significance over the rest of the experiment.

These results would suggest that increased *LHY* expression reduces the effect of osmotic stress on average seedling area and by implication, seedling growth. However, this needs to occur over a high enough threshold above the wild type level, as intermediate levels of expression performed worse (*pLHY1* and *pLHY2*).

6.1.5 Mis-expression of circadian clock genes *LHY*, *CCA1* or *TOC1* results in altered response to strong drought shock treatment

Having observed an effect on germination we tested whether LHY could also affect plant growth in drought shock conditions. We also included other circadian clock mutant lines in order to determine whether *TOC1* or *CCA1* could play a role in the drought stress phenotype. These were included as *TOC1* mis-expression had already been shown alter stomatal aperture and plant survival rates and in Chapter 5 we revealed that the LHY homolog CCA1 bound to many of the same components of the ABA signalling pathway. Seedlings were sown on soil in a randomised configuration. Both control and drought condition trays received water every 3 days for the first 14 days of growth, with watering being withheld entirely from the drought condition thereafter. Over the following 13 days aerial photographs were taken daily and analysed using ImageJ to calculate rosette surface area.

In the WT (Ler), *lhy-11*, *pLHY1* and *pLHY2* lines the drought condition began to show significantly reduced ($\leqslant 0.001$) rosette surface area after 8 days without water (see Figure 6.4). In comparison *LHY-OX* required 10 days and *pLHY3* required 11 days for a similarly significant difference to emerge between the watered

and drought conditions. Reduction in rosette surface area in the drought condition, indicating wilting, began to occur after 10 days in the *lhy-11* line and after 11 days in the WT (Ler), *pLHY1*, *pLHY2* and *pLHY3* lines. *LHY-OX* did not show a sustained reduction in rosette surface area.

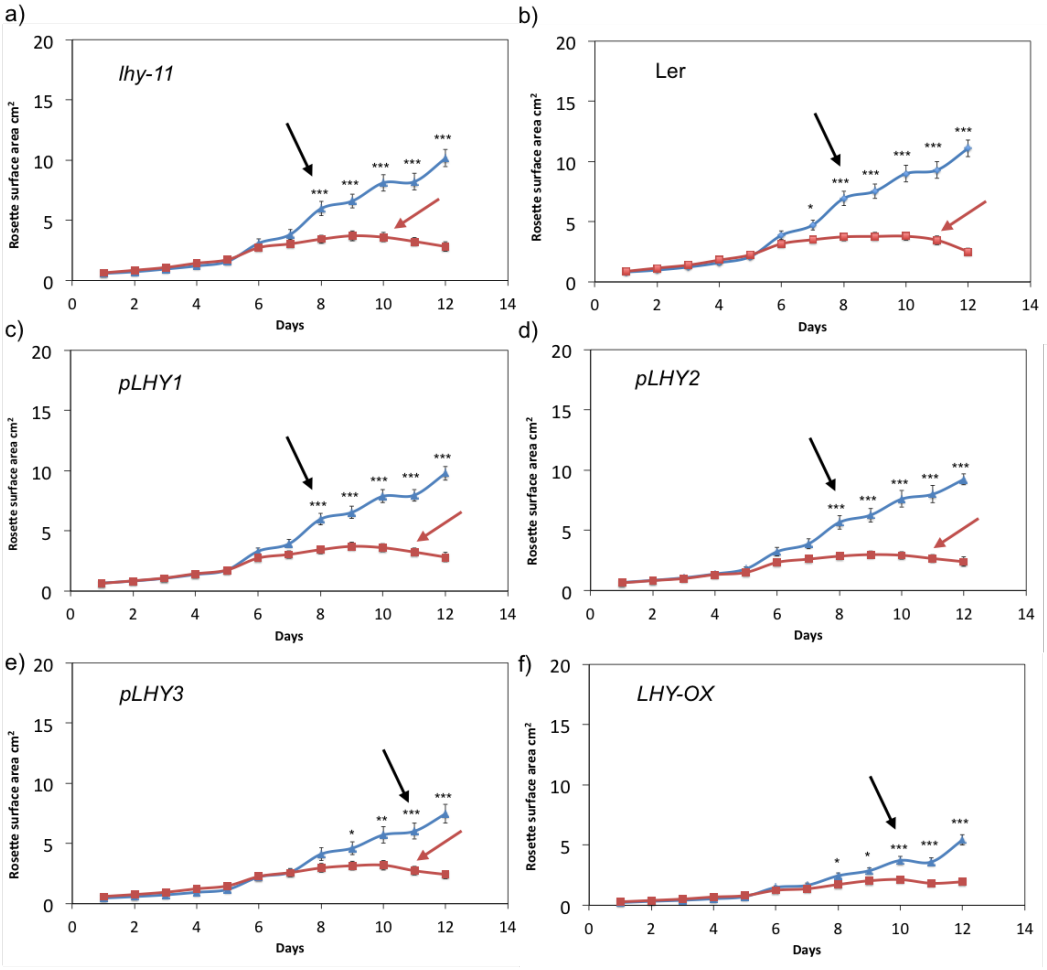

Figure 6.4 – Increased LHY levels lead to an increased performance in drought conditions in the context of rosette surface area. Seedlings were sown on soil in a randomised configuration. Both control and drought condition trays received water every 3 days for the first 14 days of growth, with watering being withheld entirely from the drought condition thereafter. Over the following 13 days aerial photographs were taken daily and analysed using ImageJ to calculate rosette surface area. Data represents the average across 24 biological replicates, with blue lines representing the watered control and red representing drought treated. Error bars indicate the Standard Error of the Mean (SEM). Results were consistent over 3 independent experiments. Asterisks indicate p-values from T-tests comparing drought and control conditions (* $p \leqslant 0.05$; ** $p \leqslant 0.01$; *** $p \leqslant 0.001$). Black arrows indicate the point at which a significant difference of $p \leqslant 0.001$ occurs between the watered and drought conditions. Red arrows indicate the point at which rosette surface area begins to enter a sustained reduction.

To further elucidate differences in rosette size we compared the percentage difference in rosette surface area between the drought and watered conditions on the final day of the experiment. This revealed that both the *LHY-OX* and *pLHY3* lines

show a statistically greater ($p \leqslant 0.05$) conservation of size with 47.2 % and 45.7 % respectively (see Figure 6.5a).

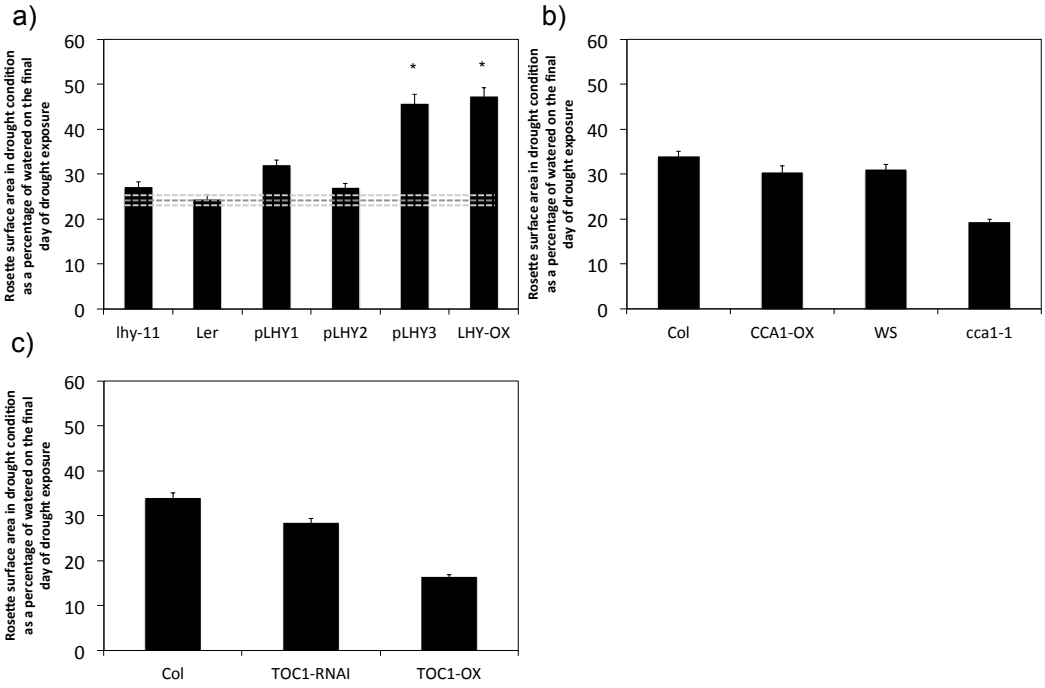

Figure 6.5 – Comparison of average rosette surface area in the drought condition as a percentage of that in the watered condition on the final day. Data represents the difference between the drought and watered conditions on the final day of the time course as plotted in figures 6.4, 6.6 and 6.7. A smaller percentage indicates that plants in the drought condition lost more rosette surface area when compared to the control condition. **a)** *LHY* mis-expressing lines. **b)** *CCA1* mis-expressing lines. **c)** *TOC1* mis-expressing lines. Error bars indicate the Standard Error of the Mean (SEM). Asterisks indicate p-values from T-tests comparing mutants to their respective wild types (* $p \leqslant 0.05$; ** $p \leqslant 0.01$; *** $p \leqslant 0.001$).

Overexpression of *CCA1* did not produce similar effects to those observed for *LHY-OX*. Drought treated plants began to diverge from the watered control after 10 days which was the same as observed in the wild type (Col) (see Figure 6.6a and c). *cca1-1* showed a greater sensitivity to drought in comparison to the wild type (WS) with significantly different ($\leqslant 0.001$) rosette sizes between the drought and watered control occurring a day before the wild type. (see Figure 6.6b and d). No significant difference was observed in either the knockout or overexpresser lines regarding conservation of rosette size in comparison to their respective wild types

(see Figure 6.5b).

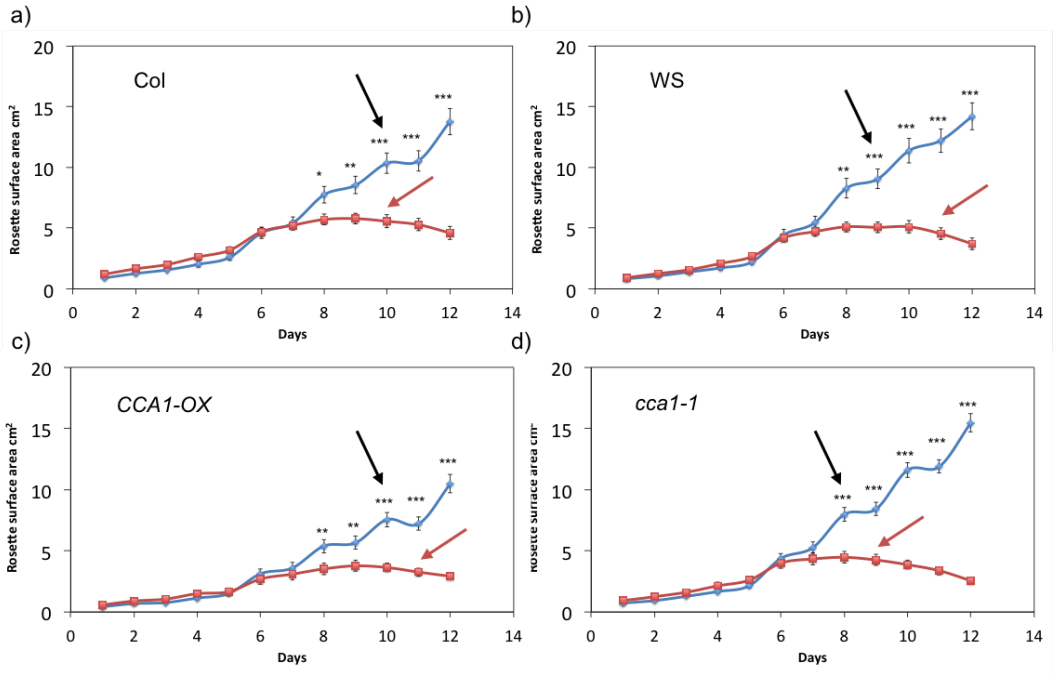

Figure 6.6 – The *cca1-1* mutant has a reduced performance in drought conditions in the context of rosette surface area. Seedlings were sown on soil in a randomised configuration. Both control and drought condition trays received water every 3 days for the first 14 days of growth, with watering being held entirely from the drought condition thereafter. Over the following 13 days aerial photographs were taken daily and analysed using ImageJ to calculate rosette surface area. *CCA1-OX* (Col) and *cca1-1* (WS). Data represents the average across 24 biological replicates, with blue lines representing the watered control and red representing drought treated. Error bars indicate the Standard Error of the Mean (SEM). Results were consistent over 3 independent experiments. Asterisks indicate p-values from T-tests comparing mutant to wild type, except in the case of **a)** and **b)** in which T-tests represent the difference between conditions in the control line (* $p \leqslant 0.05$; ** $p \leqslant 0.01$; *** $p \leqslant 0.001$). Black arrows indicate the point at which a significant difference of $p \leqslant 0.001$ occurs between the watered and drought conditions. Red arrows indicate the point at which rosette surface area begins to enter a sustained reduction.

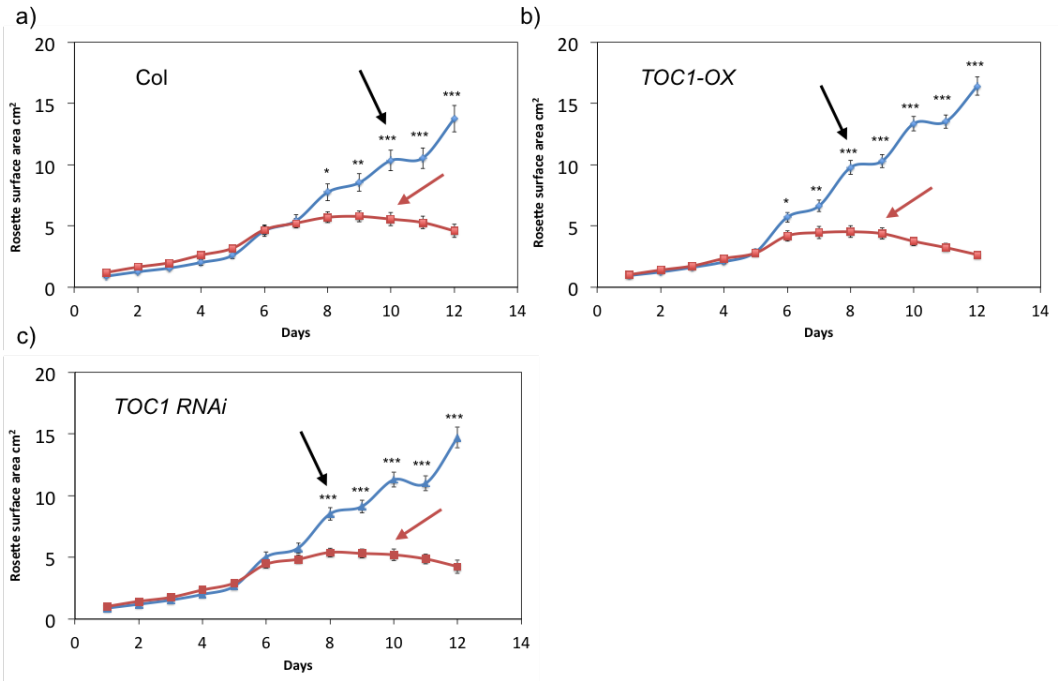

Figure 6.7 – Rosette surface area of TOC1 mis-expressing lines over time in drought and watered conditions. Seedlings were sown on soil in a randomised configuration. Both control and drought condition trays received water every 3 days for the first 14 days of growth, with watering being held entirely from the drought condition thereafter. Over the following 13 days aerial photographs were taken daily and analysed using ImageJ to calculate rosette surface area. Data represents the average across 24 biological replicates, with blue lines representing the watered control and red representing drought treated. Error bars indicate the Standard Error of the Mean (SEM). Results were consistent over 3 independent experiments. Asterisks indicate p-values from T-tests comparing mutant to wild type, except in the case of **a)** in which T-tests represent the difference between conditions in the control line. (* $p \leqslant 0.05$; ** $p \leqslant 0.01$; *** $p \leqslant 0.001$). Black arrows indicate the point at which a significant difference of $p \leqslant 0.001$ occurs between the watered and drought conditions. Red arrows indicate the point at which rosette surface area begins to enter a sustained reduction.

Overexpression of *TOC1* resulted in greater sensitivity to drought stress with significantly different rosette size observable between the watered and drought conditions after 6 days (8 days at $p \leqslant 0.001$), compared to 8 (10 at $p \leqslant 0.001$) days respectively for the wild type (see Figure 6.7). In addition rosette surface area reduction was observed 1 day earlier than that of the wild type. *TOC1-OX* also has the lowest percentage conservation of rosette size of all lines screened (see Figure 6.5c). This would be supported by the finding that ABA mediated responses are impaired in

this line as evidenced by the failure of stomatal closure (Legnaioli et al., 2009). Reduction of *TOC1* expression in the TOC1-RNAi line did not alter the point at which differences began to emerge between drought and control conditions in comparison to the wild type but did lead to differences of a higher significance 2 days earlier. Rosette surface area reduction was not different from the wild type, both starting to decline after 10 days drought treatment.

Fresh weights were measured on the final day of the experiment. Significant differences were found between drought and control conditions for all genotypes (see figure 6.8). However, the *LHY-OX* line showed the least reduction in weight, retaining a significantly ($p \leqslant 0.05$) higher percentage of its original fresh weight. Interestingly, *pLHY3* did not show significant fresh weight conservation. This is inconsistent with the perviously observed significant conservation of rosette surface area (see figure 6.5). This may indicate that whilst *pLHY3* is more resilient to the affect of drought stress on growth rate, it is not as effective in retaining moisture, leading to reduced fresh weight measurements. TOC1-OX showed the largest percentage difference between the conditions. No significant difference was observed for the *CCA1* mis-expressing lines.

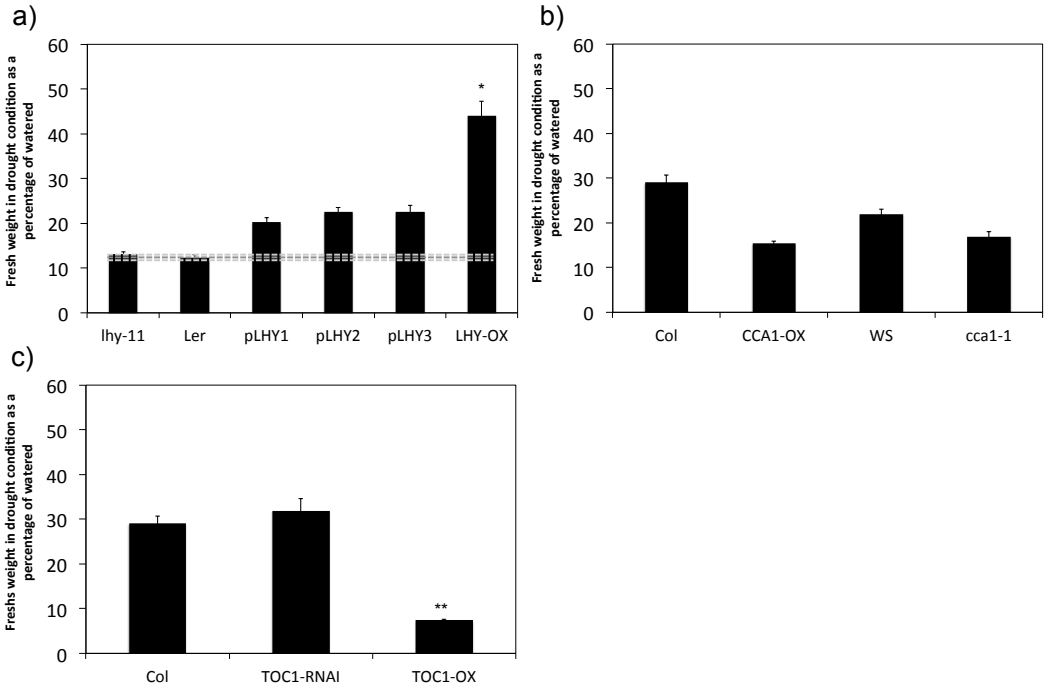

Figure 6.8 – Average fresh weight percentage conservation between drought and control conditions Data represents the average percentage conservation in dry weight measurements across 24 biological replicates with results consistent over 3 independent experiments. Asterisks indicate p-values from T-tests comparing watered and drought conditions (* $p \leqslant 0.05$; ** $p \leqslant 0.01$; *** $p \leqslant 0.001$).

6.1.6 Increased *LHY* expression results in increased performance under controlled drought and osmotic stress conditions

Having observed effects of circadian clock gene mis-expression in response to drought shock we investigated whether similar effects would occur in response to strictly controlled, *mild* drought or *mild* osmotic stress treatment. To achieve this we used an automated Phenopsis system at the CropScience facility in Ghent, Belgium. This system allowed robotic control over watering which was performed twice daily to keep pots at a stipulated target weight correlating to a specific soil water capacity (SWC) percentage (Granier et al., 2006). The lines included in this screen were as follows: Landsberg, Columbia, Wassillewskija, *LHY-OX*, *lhy-11*, *pLHY1, 2* and *pLHY3*. An extra Col-0 line from Bayer stock was included to fill the experimental

blocks. Plants were grown for 14 days after stratification and kept at 50 % SWC before stress conditions were initiated, 20 % SWC for mild drought or 150mM NaCl for mild osmotic stress. The control condition continued to receive watering to maintain 50 % SWC for the duration of the experiment. Aerial photographs for both visible and Infrared (IR) spectra were taken on the last day of controlled watering and the first day of effective stress conditions. Rosette surface areas were computed using a Bayer developed pipeline in the LemnaTec image analysis platform.

The robotic watering protocol was confirmed as successful by calculating averaged pot weight percentages in each condition over time (see Figure 6.9a). As can be seen, effective stress conditions were reached around 21 days after stratification coinciding with the initiation of daily imaging. Leaf surface temperature was measured as a proxy for stomatal opening. Therefore, it was important to determine that the growth chamber provided consistent environment across all experimental blocks so as to rule out position as a factor in the reported leaf surface temperature. Average leaf surface temperature across the course of the experiment was plotted for each plant on a grid representing the pot position in the growth chamber. No correlation was observed between pot position and temperature (see Figure 6.9b).

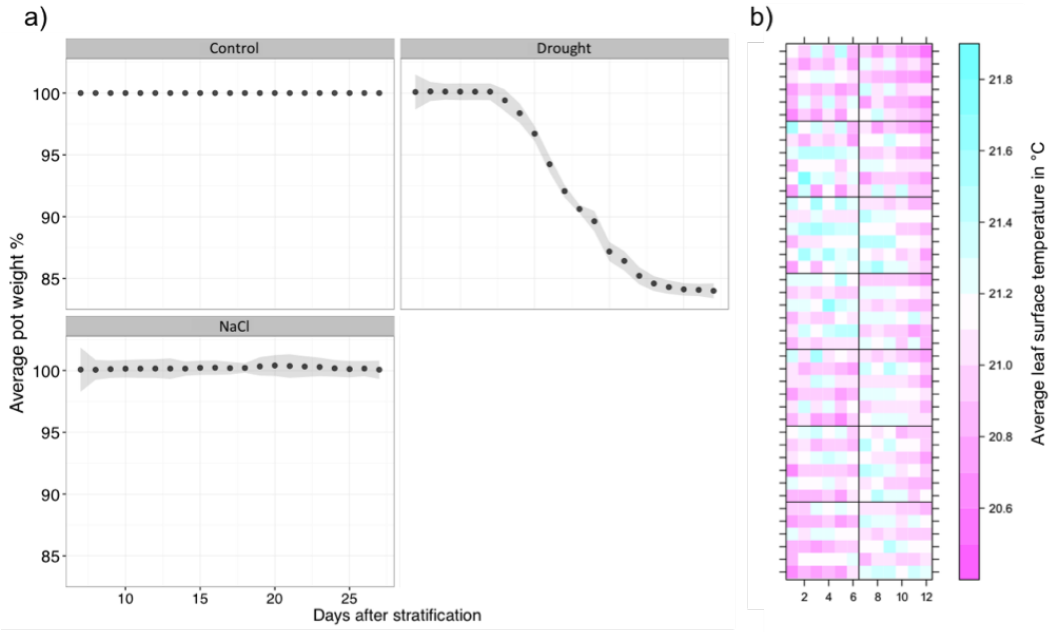

Figure 6.9 – Phenopsis control plots to verify successful growth and treatment conditions. a) Averaged pot weights were calculated as a percentage of the control condition. Data are averaged from 14 biological replicates with standard error indicated by the grey ribbon. **b)** Averaged leaf surface temperature for each plant over the whole time course. 14 blocks of 36 plants correspond to the 504 individual plants across experimental blocks, positions correlate with the randomised design configuration.

All watered controls outperformed stress treatments as expected indicating successful treatment (see figure 6.10). Drought was the strongest stimulus affecting rosette surface area resulting in significantly smaller rosette surface areas. All genotypes were mildly affected by salt stress alone, performing worse than the control but better than the drought treatments. However, this only resulted in significantly smaller rosette sizes for the Landsberg, *lhy-11* and *pLHY1* lines.

As had been previously observed under drought shock, the *LHY-OX* line showed no significant differences between treatments suggesting similar performance under all conditions tested. This trend was also observed in *pLHY2* and *pLHY3* with both lines showing minimal differences between treatments. *pLHY3* performed most similarly to that of *LHY-OX* with drought treatment only causing significant reduction

in rosette size in comparison to the watered control on the final 2 days of stress. *lhy-11* performed better than the wild type (Ler) in presence of salt stress with only two time points being significantly different from the watered control.

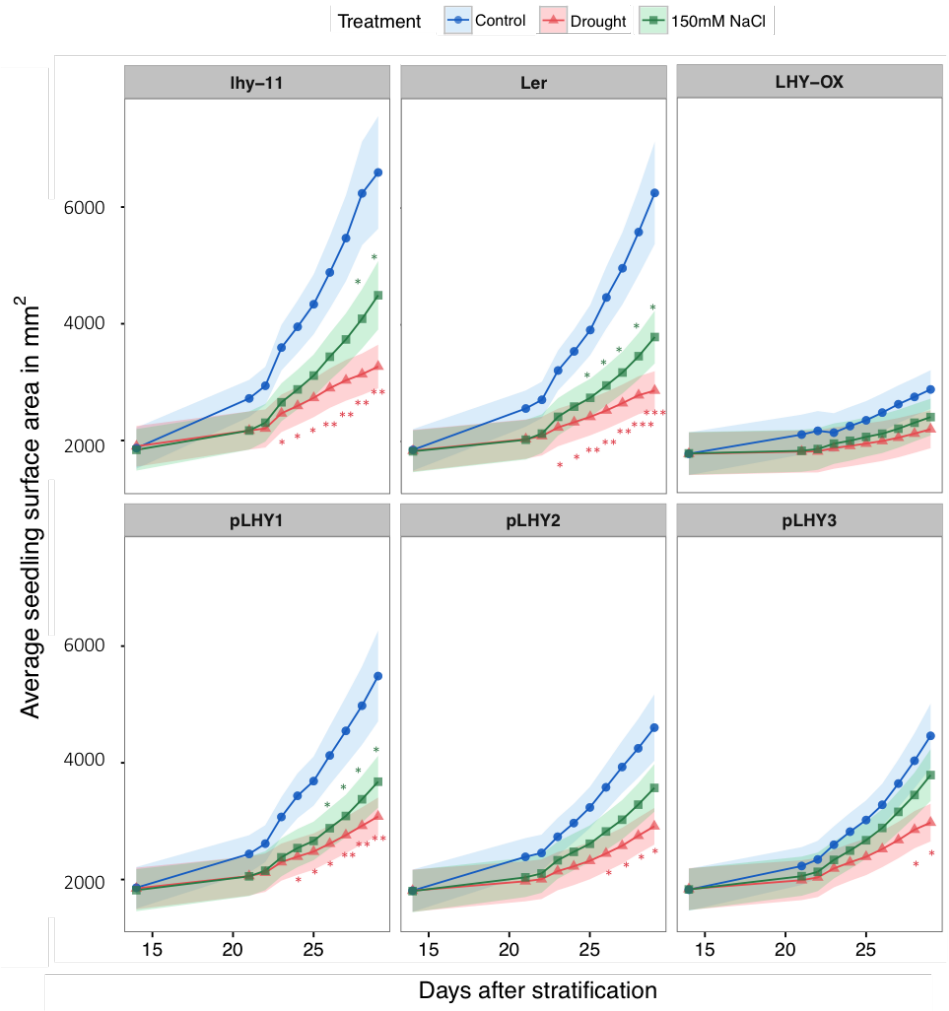

Figure 6.10 – Rosette surface area over time across all genotypes and treatments. Seedlings were grown for 14 days under control conditions and watered at 50 % SWC. After this period, growth continued for 14 days with the drought condition receiving 20 % SWC and osmotic stress condition receiving 150 mM NaCl at 50 % SWC. Rosette surface area was calculated based on imaging data collected during effective stress treatment. Data represents the mean of 14 biological replicates per genotype per treatment. Standard deviation is indicated by the coloured ribbon for each treatment. Asterisks indicate p-values from T-tests comparing experimental treatments to the control condition within each genotype. Green asterisks indicate the significance between the NaCl and control condition with red for the difference between drought and control (* $p \leqslant 0.05$; ** $p \leqslant 0.01$; *** $p \leqslant 0.001$).

Comparison of rosette surface area conservation on the final day of the experiment revealed that *pLHY3* and *LHY-OX* performed significantly ($\leqslant$0.05) better than the wild type (50.3 %) under salt stress retaining 76.6 % and 77.4 % of their watered size respectively (see figure 6.11a and b). Under drought stress *pLHY2*, *pLHY3* and *LHY-OX* (which was the most significant) retained a significantly higher percentage of rosette surface area in comparison to the wild type (30.3 %) with 46.2 %, 61.7 % and 55.5 % respectively.

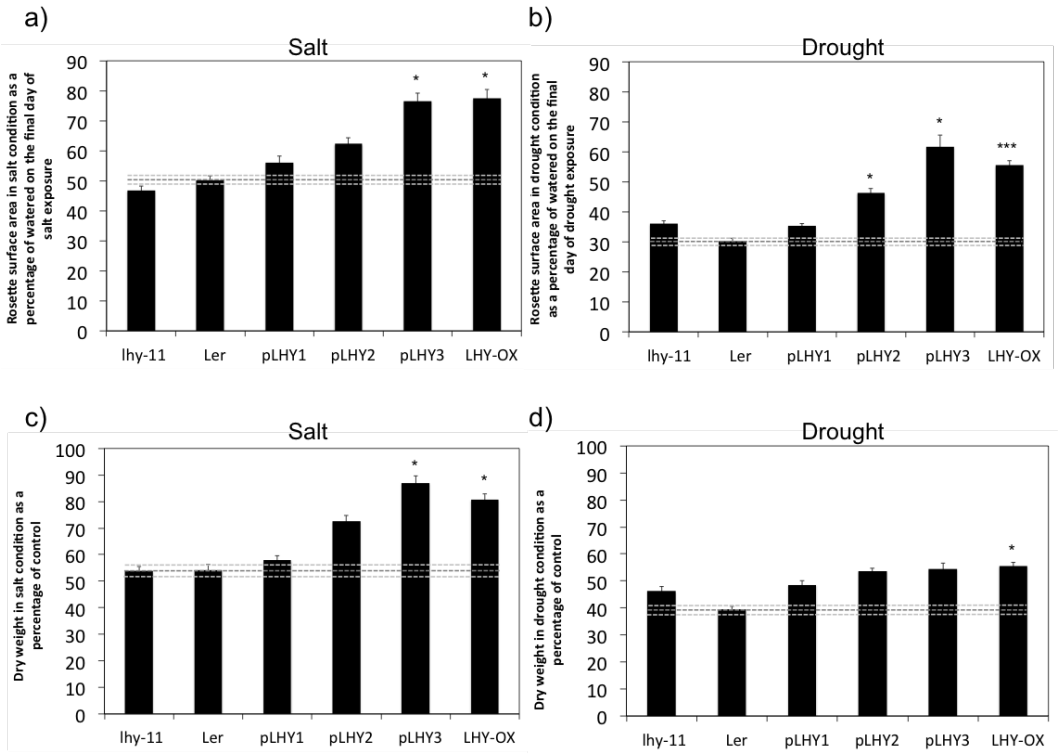

Figure 6.11 – Comparison of average rosette surface area and dry weight measurements as a percentage of that in the control condition on the final day. **a) and b)** Data represents the difference in rosette surface area between the drought and watered conditions on the final day of the time course as plotted in figure 6.10, A smaller percentage indicates that plants in the drought condition lost more rosette surface area when compared to the control condition. **c) and d)** Data represents the average percentage difference in dry weight measurements across 14 biological replicates. Asterisks indicate p-values from T-tests comparing genotypes to their respective wild type (Ler) (* p$\leqslant$0.05; ** p$\leqslant$0.01; *** p$\leqslant$0.001).

Average surface temperature was measured via infrared thermography (see Figure

6.12). Increased surface temperature is highly correlated with stomatal aperture and rises when stomata are closed. Interestingly there was a large and highly significant increase in surface temperature in drought treatments across all genotypes which peaked during the first few days of the effective stress period. This was not observed for the salt stress treatment which was not significantly different from the control at any time point in any line.

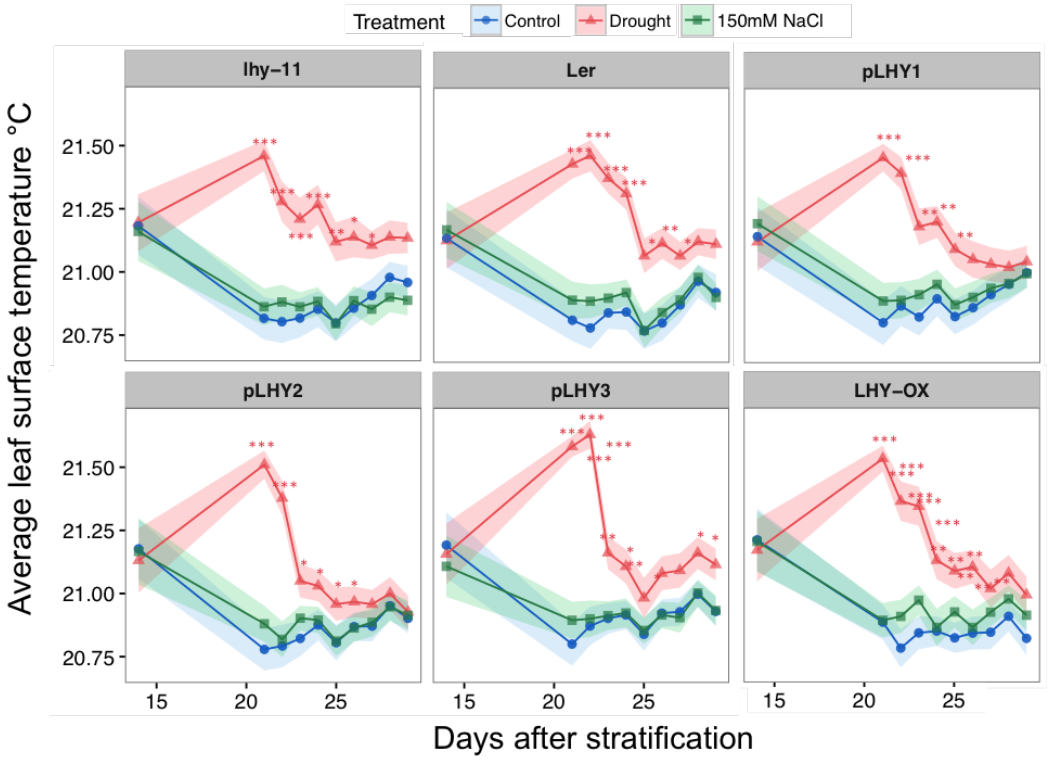

Figure 6.12 – Average rosette surface temperature over time grouped by genotype. Seedlings were grown for 14 days under control conditions and watered at 50 % SWC. After this period, growth continued for 14 days with the drought condition receiving 20 % SRC and osmotic stress condition receiving 150 mM NaCl at 50 % SRC. Rosette surface temperature was measured via infrared thermography during effective stress treatment. Data represents the mean of 14 biological replicates per genotype per treatment. Standard deviation is indicated by the coloured ribbon for each treatment. Asterisks indicate p-values from T-tests comparing experimental treatments to the control condition within each genotype. Green asterisks indicate the significance between the NaCl and control condition with red for the difference between drought and control (* $p \leqslant 0.05$; ** $p \leqslant 0.01$; *** $p \leqslant 0.001$).

No significant differences were observed between genotypes in either control or stress

conditions suggesting that stomatal control occurs as normal in response to drought and salt stress in these lines (see Figure 6.13).

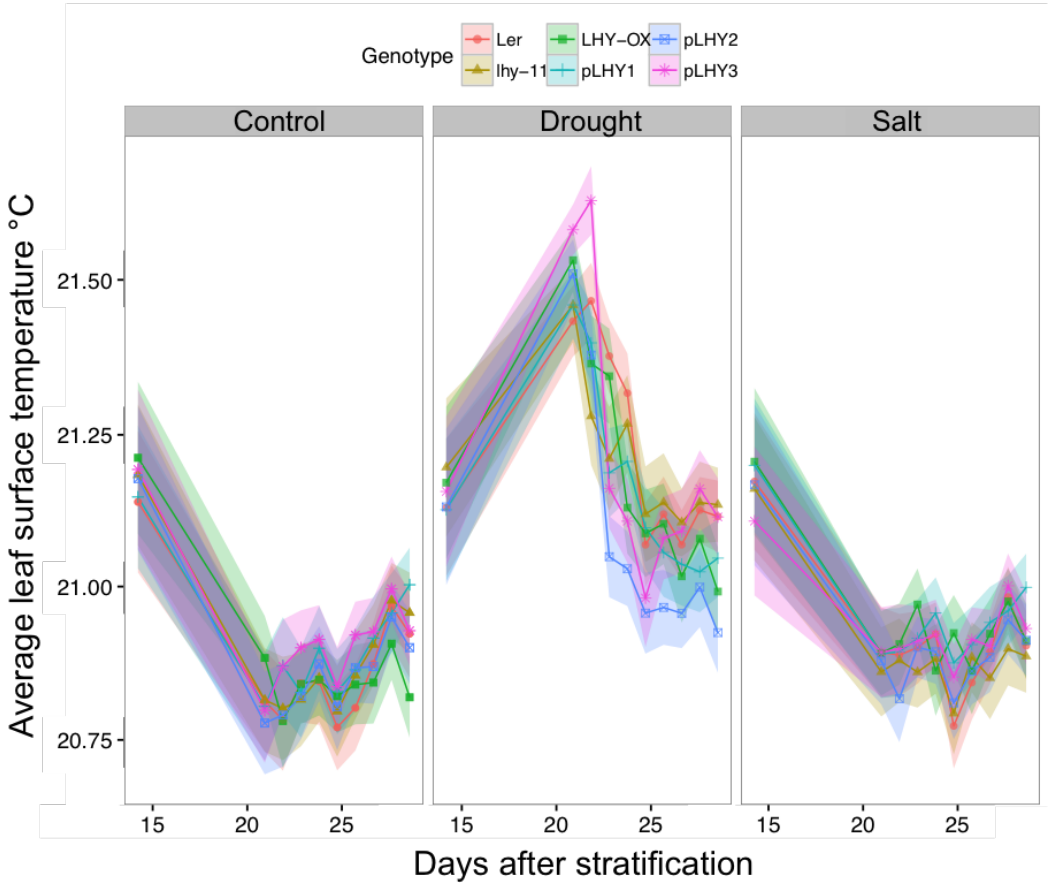

Figure 6.13 – Average rosette surface temperature over time grouped by treatment. Seedlings were grown for 14 days under control conditions and watered at 50 % SWC. After this period, growth continued for 14 days with the drought condition receiving 20 % SRC and osmotic stress condition receiving 150 mM NaCl at 50 % SRC. Rosette surface temperature was measured via infrared thermography during effective stress treatment. Data represents the mean of 14 biological replicates per genotype per treatment. Standard deviation is indicated by the coloured ribbon for each treatment.

Dry weight measurements were taken on the final day after a period of oven drying (see Figures 6.11c and d). Biomass loss was less severe in response to salt stress than drought. *pLHY3* and *LHY-OX* both performed significantly ($\leqslant 0.05$) better than the wild type (54.2 %) conserving a greater percentage of their watered biomass with 78 % and 80.8 % respectively. Under drought stress only *LHY-OX* performed

significantly ($\leqslant$0.05) better than the wild type (39.5 %) conserving 55.6 %.

In order to quantify potential phenotypic differences in onset and severity of senescence, colour analysis was performed averaging Red, Green and Blue (RGB) values for images taken on the final day of the growth. This provided an indication on the degree of colour change in response to each treatment condition for each genotype (see Figure 6.14 and Table 6.1). As it can be difficult to interpret changes in multivariate data, the Euclidean distance for each condition relative to the control treatment is represented in Figure 6.15. Landsberg showed a large deviation from the control colour in response to stress, with all three treatments producing a response of similar intensity. *lhy-11* performed similarly to the wild type. *LHY-OX*, however, had a tightly clustered set of colour measurements for all conditions indicating a possible delayed response or insensitivity to stress in the context of senescence. As *LHY-OX* differs from both *lhy-11* and Landsberg in the control condition, this indicates that overexpression of *LHY* has a constitutive effect on colouration and may reflect a pre-emptive priming for stress conditions. All three complemented lines showed a reduced colour response for all treatments when compared with the wild type although *pLHY3* showed the least.

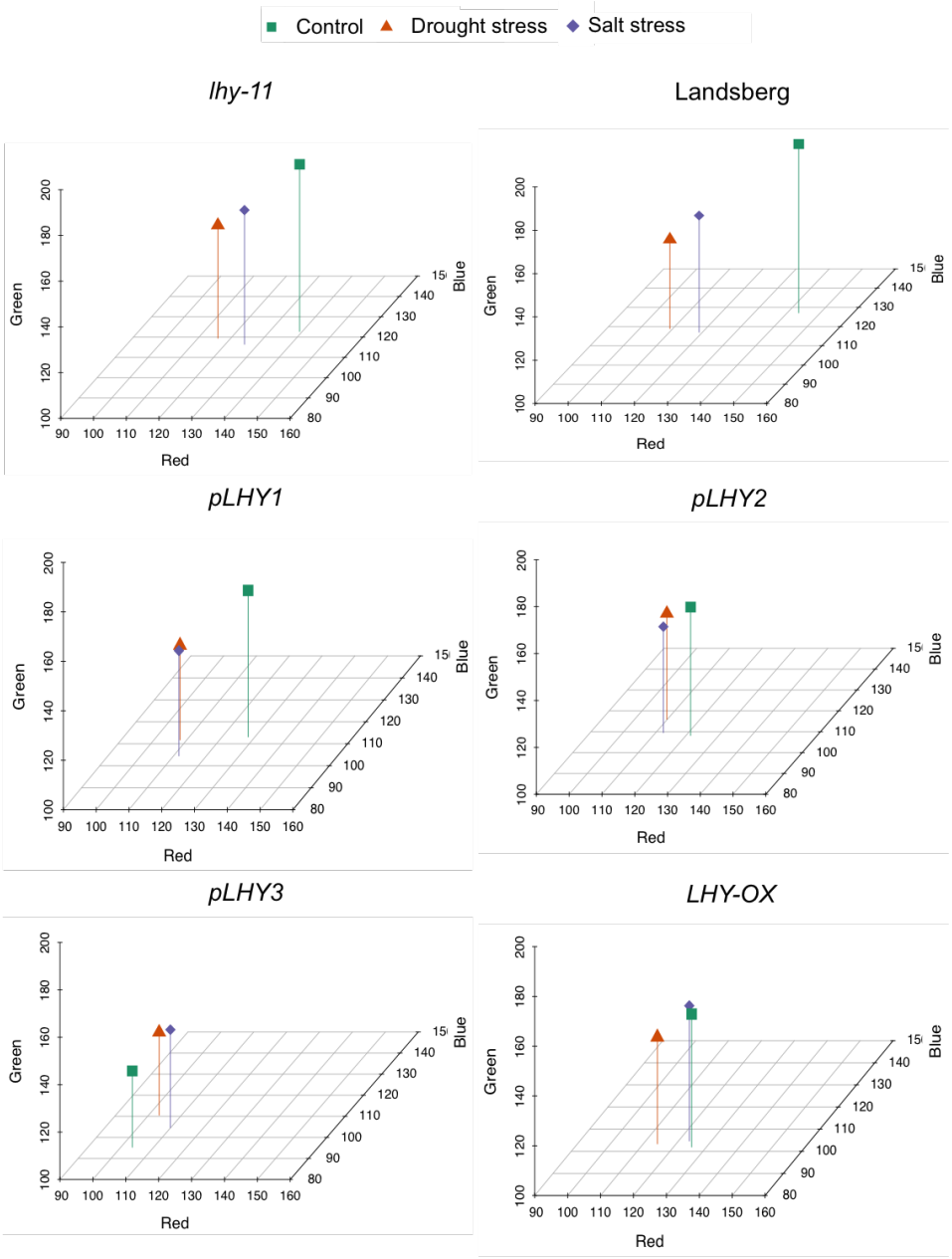

Figure 6.14 – Averaged RGB colour values of rosettes on the final day, represented in 3D space. Data represents means from 14 biological replicates per genotype per treatment measured after 14 days of stress and 29 days of total growth. Standard Error of the Mean for each is provided in table 6.1

	Control			Drought			Salt		
Genotype	Red	Blue	Green	Red	Blue	Green	Red	Blue	Green
Ler	0.97	1.24	0.54	2.83	4.00	3.59	3.22	3.57	3.70
lhy-11	0.65	0.59	0.62	2.16	3.16	2.76	1.80	1.69	2.01
LHY-OX	3.24	2.89	2.78	1.89	2.18	2.69	3.46	3.25	3.77
pLHY1	3.25	3.38	3.83	1.45	1.85	1.95	2.85	3.26	3.64
pLHY2	2.48	2.52	2.42	2.07	2.76	2.73	2.02	1.96	2.40
pLHY3	2.73	3.05	3.69	1.65	1.94	2.14	2.18	2.62	2.78

Table 6.1 – SEM of RGB colour values on the final day Data represents Standard Error of the Mean (SEM) for each RGB colour channel as plotted in figure 6.14.

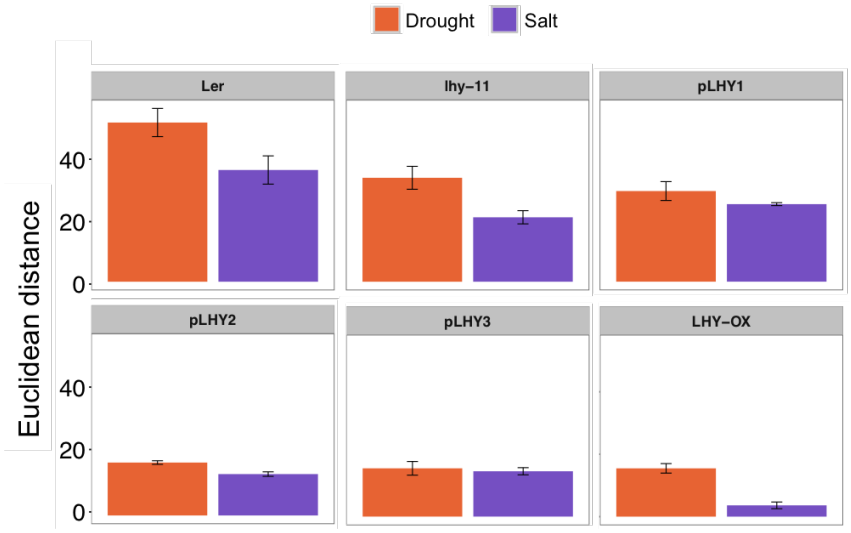

Figure 6.15 – Euclidean distance for colour changes in response to stress conditions. Data represents means from 14 biological replicates per genotype per treatment measured after 14 days of stress and 29 days of total growth. Distances for each experimental condition are calculated relative to the control position for each genotype which serves as an origin.

Having identified that all experimental conditions produced some level of colour change in rosettes of all genotypes, we performed colour analysis over time to investigate on what time scale these changes occurred. This revealed that there were no significant differences in the rate or time of onset of colour change between genotypes (see Figure 6.16). Onset occurred after approximately 21 days, the time at which the treatment conditions were becoming effective, and reached a stable plateau after 2 days. This was a similar time scale to that observed for changes in leaf surface temperature (see Figure 6.12). Natural developmental changes in colour may occur around 3 weeks after stratification as the control lines exhibited similar responses. This could mask finer treatment specific differences between lines. However stress treatment did not result in early or delayed onset of this universally observed colour change.

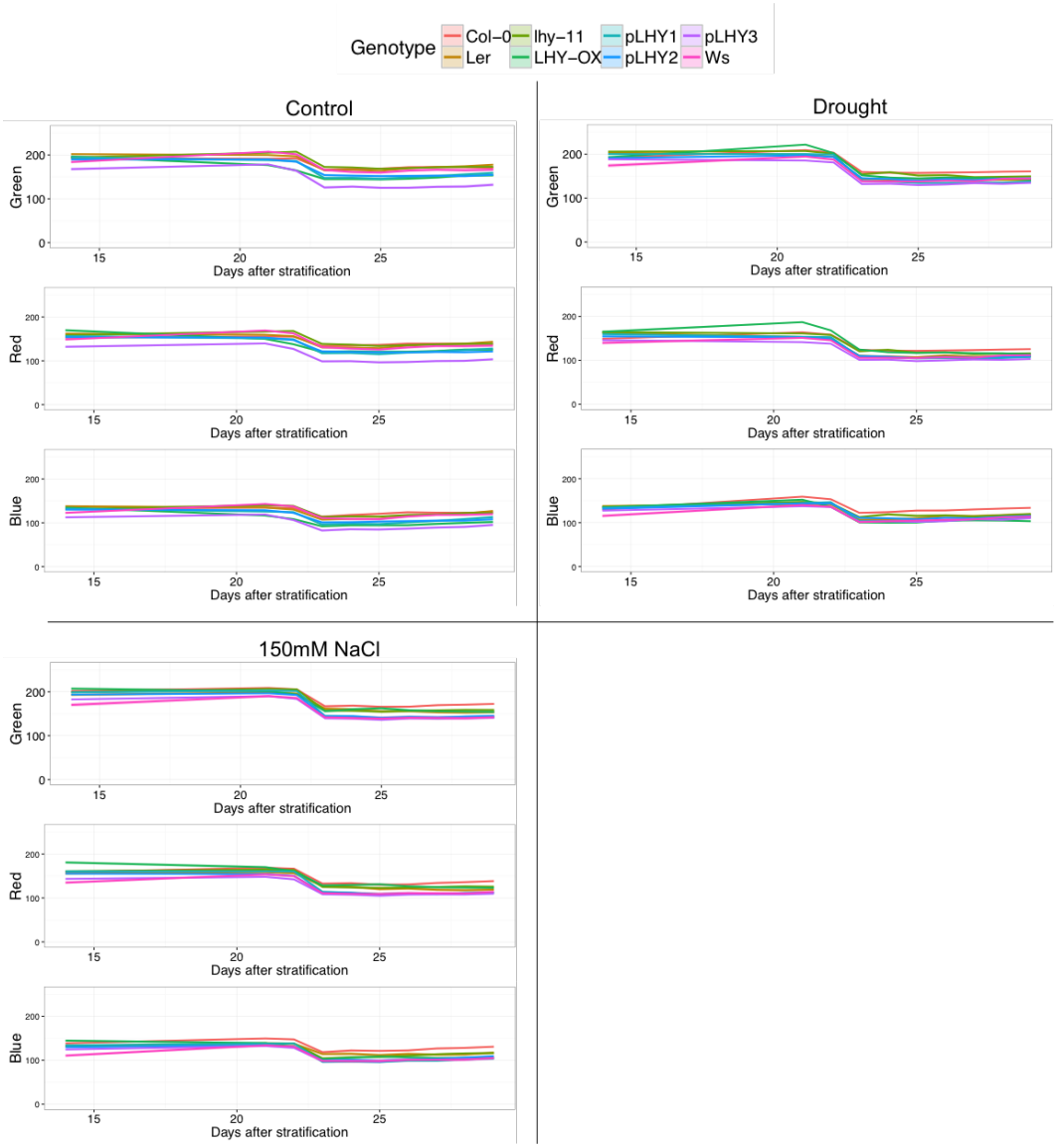

Figure 6.16 – Average RGB values plotted over time across genotypes and treatment conditions Data represents means from 14 biological replicates per genotype per treatment measured daily across the stress treatment period. Standard deviation is indicated by the coloured ribbon for each treatment.

6.1.7 Discussion

Results presented in Chapter 5 demonstrated that increased *LHY* expression resulted in heightened transcriptional responses to ABA and mitigated the negative effects of ABA on growth and seed germination. As identical responses are observed

under osmotic stress, these enhanced responses could be the reason for stronger mitigation of the effects of osmotic stress. For example, we revealed that wild type seedlings in the presence of 300 mM sorbitol had significantly reduced average seedling area and that these effects were abolished in the *LHY-OX* and *pLHY3* lines. This improved tolerance was not observed in either *pLHY1* or *pLHY2*, performing worse than the wild type. A potential explanation of this could be that as overexpression of *LHY* results in reduced ABA biosynthesis this effect is offset via increased induction of stress responsive genes generating a greater overall tolerance in the *LHY-OX* line. Mild over expression of *LHY* in *pLHY1* and *pLHY2* may not sufficiently promote the induction of protective gene expression so as to outweigh suppression of ABA biosynthesis. This indicates that there may be a threshold over which *LHY* overexpression has to pass in order to produce improved plant performance under stress conditions. Chapter 5 also showed that reduced *LHY* expression resulted in reduced ABA responsive gene expression in comparison to the wild type. This is not reflected in the average seedling area of the *lhy-11* line which, in the presence of sorbitol, performed better than the wild type. Interestingly, this improved performance was not supported by either the drought shock, mild drought or mild osmotic stress data sets. This indicates that any potential benefits of reduced *LHY* expression during early seedling growth do not confer lasting advantages in regards to conserved rosette surface area or biomass under stress, instead performing similarly to the wild type.

Using drought conditions we have also shown that mis-expression of *LHY* can improve drought tolerance. As a positive control for our experimental conditions, we were able to confirm previously reported results which had demonstrated an increased sensitivity to drought stress in the TOC1-OX line (Legnaioli et al., 2009). This was achieved using an alternative measurement (rosette surface area) than that which was already published (survival rate), providing further characterisation of

this response. TOC1 overexpression reduced the time taken for drought stress to cause significant reduction in rosette surface area with rosettes conserving significantly lower biomass than the wild type. We did not observe improved performance in the TOC1-RNAi line, instead it matched that of the wild type. This may be because the drought shock treatment was too severe, withholding water entirely.

Increased *LHY* expression in drought shock, mild drought stress and osmotic stress treatments increased conservation of rosette surface and biomass. Differences were not observed for mutants mis-expressing *CCA1* which is interesting as *CCA1* is believed to act redundantly to *LHY* as a close homolog and CCA1 was shown to bind to many of the same ABA signaling components as LHY. This suggests that LHY is key for regulating ABA-dependent and independent stress responses. In light of this, the altered performance of the *TOC1* mis-expressing lines could be via the effects on *LHY* expression. As TOC1 negatively regulates *LHY* (Gendron et al., 2012) the TOC1 RNAi line, which is less sensitive to drought stress, would also have increased LHY levels.

As mentioned in Chapter 5, although *LHY-OX* has a developmental phenotype with elongated petioles and hypocotyl, producing a rosette with smaller surface area during early stages of growth perhaps reducing transpirational loss, as these plants mature they resemble the wild type and become much more robust. Mild over expression ameliorates this morphological phenotype yet still provides increased osmotic stress tolerance. Furthermore, infrared thermography was performed as a proxy measurement for stomatal aperture and thus a proxy for transpirational water loss. Lower leaf surface temperatures were interpreted to correspond to a greater presence of stomata in an open configuration leading to increased transpiration. This negative correlation has been reported in numerous studies and this technique has been used to isolate *Arabidopsis* mutants defective in stomatal regulation (Costa

et al., 2013; Munns et al., 2010; Merlot et al., 2002). No differences were detected between the mis-expressing *LHY* lines suggesting that *LHY-OX* was not improved via enhanced stomatal sensitivity.

Increased *LHY* expression was also shown to improve plant performance in drought conditions through colour analysis. In an attempt to quantify differences in senescence in response to osmotic stress, RGB colour profiles were compared for *LHY* mis-expressing lines. In response to abiotic stress energy is diverted away from cell elongation in the leaves and results in an increase in the number of cells per unit leaf area and darker leaf colour (Strawn et al., 2015). Instead available energy is redirected to produce osmolytes in order to compete with a reduced water potential in the soil. We showed that an increase in *LHY* resulted in a reduction in colour change in response to stress. LEAs are a family of small, highly hydrophilic proteins which accumulate in response to abiotic stress such as dehydration (Popova et al., 2015). In Chapter 5 we showed that increased *LHY* expression resulted in higher levels of *LEA7* expression in response to ABA application and could play a role in mitigating senescent effects.

Together the results presented within this chapter demonstrate that LHY plays an important role in regulating responses to drought stress and that mild overexpression of LHY results in improved drought tolerance.

Chapter 7

General Discussion

The world-wide population is predicted to increase from 7.2 billion to between 9.6 and 12.3 billion by the year 2100 (Gerland et al., 2014). With an increasing global population comes an increasing demand on global food productivity. This problem is exacerbated by world-wide crop losses due to extremes of weather and the limited amount of land suitable for cultivation (European Union, 2007; Eischeid et al., 2014; Boyer, 1982). As a result there is an urgent need for the production of crop varieties with increased tolerance to abiotic stress.

Current approaches in genetic engineering for increased stress tolerance in plants have centered around the overexpression of stress responsive transgenes. This has had many successful results a few of which have been approved for commercialisation (Lawlor, 2013; Cabello et al., 2014; Waltz, 2014). However, a negative side effect that can occur as a result of this approach is that the constitutive overexpression of stress responsive genes can lead to growth arrests, decreased yields or other unwanted pleiotropic effects (Hussain et al., 2011). These problems can be reduced via the use of stress responsive promoter regions, restricting expression to times when it would be beneficial. Nevertheless, genetic manipulation of master regulators could potentially be a more powerful approach due to the broad spectrum of downstream

mechanisms that could be affected.

Previous research has revealed a web of regulatory connections between abiotic stress responses and the circadian clock. The significance of this is that stress can be perceived and integrated into a central control system whereby acclimatisation and anticipation become possible. Despite abiotic stress not often occurring as a predictable daily phenomenon, genes associated with heat, cold and drought stress have tightly clustered phases of expression across a 24 hour period. For example, heat stress responsive genes are upregulated during the mid-day and afternoon when temperatures can be expected to reach their highest (Grundy et al., 2015). Circadian control also enables regulation of the mechanisms through which stress is perceived, allowing for fine-tuning of the system's sensitivity and timing of sensitivity. Due to the sessile nature of plants, this is an important mechanism by which the effects of external environmental stress can be managed.

In this thesis we have demonstrated this fine-tuning as we have shown that ABA hormone induction in response to stress is time of day dependent, peaking at the night transition. Circadian regulation of the quantity of ABA produced in response to a stress stimulus may function as an effective way to control the overall downstream stress response in the plant, perhaps allowing for some level of override during the day in which ambient heat fluctuations could otherwise trigger stomatal closure and throttle photosynthesis.

Furthermore we have identified the core circadian clock gene *LHY* as having a strong role in both the integration of incoming abiotic stress responses into the circadian clock and in the regulation of abiotic stress perception. This role was not entirely unprecedented, as mis-expression of the evening associated core clock gene *TOC1* had previously been shown to bind to the putative ABA receptor, ABAR,

and disrupt stress induced stomatal closure (Legnaioli et al., 2009). However, we have demonstrated via ChIP-seq data analysis that LHY is potentially the most important clock component in regulating response to ABA as it bound directly to the promoter sequence of many of the canonical ABA signalling pathway components. In addition, ABA regulated genes were found to be over represented among direct LHY binding targets. Increased *LHY* expression led to greater abiotic stress responsive gene induction and greater plant performance under stress conditions. Interestingly, germination assays revealed that increased *LHY* expression also decreased the perceptive sensitivity to abiotic stress. This is important as overexpression of *LHY* appears to allow for targeted reinforcement of stress tolerance, mitigating the negative effects associated with hypersensitivity and promoting increased tolerance through enhanced protective gene expression.

As LHY is a central oscillator component, clamping, through strong overexpression of *LHY* results in an arrhythmic clock in constant light (Kim et al., 2003). In response to long-term stress, we have observed that circadian rhythms become damped and show no recovery upon the removal of the stress stimulus over the short term. Similar observations have been published in response cold stress in *Arabidopsis* (Bieniawska et al., 2008) and to drought stress in both soybean (Marcolino-Gomes et al., 2014), and barley (Habte et al., 2014). This damping could be significant as an internal stress response strategy whereby the removal of large changes in amplitude of clock genes across the day disrupts any existing predisposition towards override of stress perception. Overexpression of *LHY* may be inducing this strategy artificially.

Lack of oscillation recovery may also aid in acclimatisation and serve as a stress 'memory'. Just like perturbing the oscillation of a pendulum results in lasting changes in its swinging pattern, acute up or downregulation of the clock genes in re-

sponse to abiotic stress would be expected to result in persistent changes in the phase or amplitude of their oscillation. This may, in turn, result in long-term changes in the expression of the stress response pathways that they regulate.

The work in this thesis suggests that manipulation of the circadian clock, a system that contains many master regulators in its core architecture, is a potential strategy for developing increased stress resistance in crops. Core clock gene homologs are present in a wide range of commercial crop species across both eudicots and monocts such as as soybean (Marcolino-Gomes et al., 2014), rice (Murakami et al., 2007), wheat (Alvarez et al., 2016) and barley (Faure et al., 2012; Habte et al., 2014; Calixto et al., 2015).

Regarding *LHY*, strong overexpression produces a morphological phenotype during early growth stages producing smaller seedlings with elongated petioles and hypocotyl. Although this does not persist in adult plants, in a field scenario this increased vulnerability during early stages of growth may render such lines non-viable. Genetic manipulation of master regulators does have the potential to cause great disruption and decrease fitness. It is important, therefore, to explore a range of mild mis-expession of such genes, gently altering the circadian function. In doing so we have observed increased plant performance in plant lines with mild overexpression (which do not have the morphological phenotype) that is comparable with that of the strong overexpressor. This demonstrated a strong potential for manipulation of circadian clock function, through *LHY*, as a novel approach to engineering increased stress tolerance.

An important avenue of future work would be to assay the performance of a crop lines overexpressing the *LHY* homolog under multiple abiotic stress conditions. This would serve as a proof of concept, to further confirm the findings presented within

this thesis and to explore translation from *Arabidopsis* into commercial crops for commercial application.

Appendix A

A.0.1 *LHY* expression in response to ABA application in the Columbia ecotype

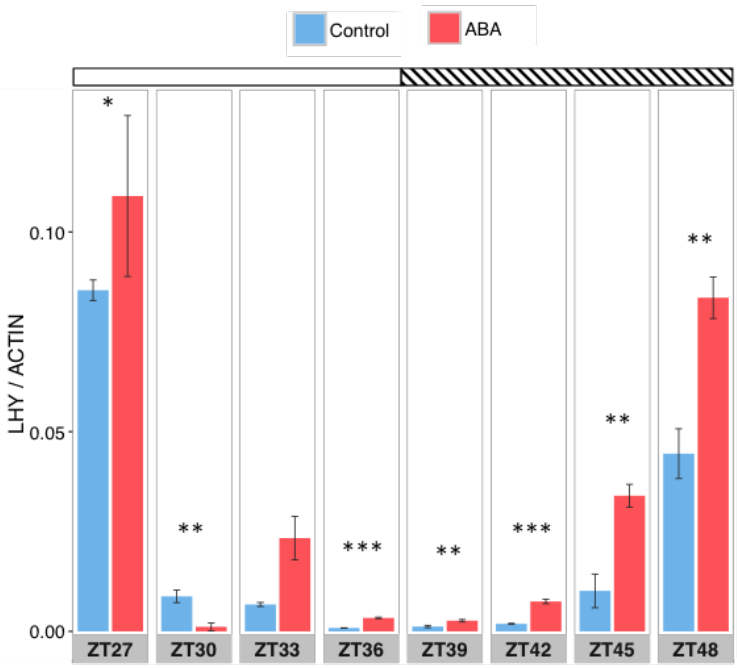

Figure A.1 – ABA induces *LHY* expression when applied at dawn in the Columbia ecotype. *Arabidopsis* seedlings (Col) were grown on MS0 for 7 days at 22°C and entrained to a 12L 12D lighting regimen before being transferred to constant light. A single application of ABA (25 μM) or vehicle (methanol) was sprayed onto plants at one of the following time points (ZT24, 27, 30, 33, 36, 39, 42, 45), harvested 4 hours after and flash frozen. **a)** *LHY* Data represents the mean of technical triplicates with error bars showing standard deviation. Results were consistent across three independent biological replicates.

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